TARGETING CAPN9/CAPNS2 ACTIVITY AS A THERAPEUTIC STRATEGY FOR THE TREATMENT OF TRANSFORMING GROWTH FACTOR BETA-INDUCED MESENCHYMAL TRANSITION AND ASSOCIATED PATHOLOGIES

by

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Abstract

High expression and/or activity of calpain proteases has been associated with the development of fibrosis, but a mechanistic explanation of their role in this process remains to be defined. In this work, we show that calpain-dependent proteolysis is required for Transforming Growth Factor β (TGFβ)-induced Epithelial-to-Mesenchymal Transition (EMT), Endothelial-to-Mesenchymal Transition (EnMT), and Fibroblast-to-Myofibroblast Transition (FMT). These related cellular processes promote the generation of myofibroblasts, a cell type that has been strongly implicated in driving fibrosis. We show that broad-spectrum calpain inhibitors can suppress EMT or FMT without affecting early TGFβ signaling events, such as phosphorylation of SMAD2. Importantly, calpain activity was also required for maintenance of an established myofibroblast fate. Overexpression of the endogenous dimeric calpain inhibitor, calpastatin, also potently suppressed TGFβ1-induced EMT. RNA interference (RNAi)-mediated knockdown of individual dimeric calpain isoforms, during TGFβ1-induced EMT, showed that only inhibition of CAPN9 or CAPNS2 could suppress generation of myofibroblasts. In epithelial cells, CAPN9 and CAPNS2 were expressed only under high TGFβ signaling conditions. Current data suggests that CAPN9 and CAPNS2 can form a heterodimer, whose activity we hypothesize is necessary for TGFβ-induced mesenchymal transition. We go on to show that expression of CAPN9 is also necessary for TGFβ1-induced cleavage of calcineurin, a known activator of FMT (Davis 2012). In mice, intratracheal delivery of siRNA targeting *Capns2* or knockout of *Capn9* was able to ameliorate bleomycin-induced lung

fibrosis. These data suggest that the selective targeting of the CAPN9/CAPNS2 heterodimer may be a powerful therapeutic strategy for the treatment of fibrotic disorders with minimal side effects.

Advisor: Hal Dietz, M.D.

Reader: Susan Michaelis, Ph.D.

Preface

The Kobayashi Maru

“I don’t believe in the no-win scenario.”

-Admiral James T. Kirk

I dedicate this thesis to my grandmother, who passed away shortly before I could finish this work. I am saddened that she could not witness this milestone in my life, but I know that she is watching over me, proud of all that I have done and will do in the future. Having lived most of my life in California, I came to Johns Hopkins with the hope of getting a different perspective on science that I could take back with me. It has been hard to be so far away from home and my mom for such a long period of time but I am thankful for having received this unique opportunity to learn. My mom has worked very hard and sacrificed very much, and has done everything in her power in order that I may find happiness in my life.

My mentor, Hal Dietz, has left a profound impression on me during my time in his lab. Teaching by example, Hal has passed onto me his voice, style, sensibilities, judgement, and spirit; all things that have made him second to none. Hal never gave up on trying to bring out the very best in me. Part of him will be with me forever, seen and expressed in the scientist that I will become. Elena Gallo, our faculty instructor, has been an endless source of wisdom for not only me, but all students in the lab. Elena also helped to edit this thesis, using her quick mind and keen analytical abilities. Our research specialist, Yi-Chun Chen,

has always been there to help in times where I was stressed and overwhelmed. I could not have done this work without her expertise and generosity. I argue that Sara Cooke is the best lab manager in all of Johns Hopkins, managing what is probably the most demanding and difficult group of scientists also at Johns Hopkins. Her years of experience, wisdom, temperance, and positive attitude have helped me through my hardest times. When I first entered the lab, postdocs Dave Loch and Christel van Erp welcomed me with open arms and I felt that they were always looking out for me. Although I have missed Dave's wit and humor, I feel he imparted some of that to me before he and Christel left. Similarly, I have grown fond of Shira Ziegler, who upon entering our lab, brought with her an infectious positive attitude, strong common sense, and willingness to help whenever possible. I'd also like to thank Mark Lindsay, who began the work on EMT in aortic aneurysm, and Nishant Patel, whose work suggested looking at calpains. I'd also like to thank Debbie Churchill, who has helped with the significant logistics that come with being a graduate student.

I'd also like to thank Susan Michaelis, the chair of my thesis committee, who took time to have long conversations with me about science and my future career. In addition, Susan took the time to read my thesis with vigor on short notice. She has been one of my strongest advocates and supporters, something I will not forget and that I will try to emulate for the next generation. David Kass also served on my thesis committee and provided incredible insight that helped to guide the direction of this research. I am also indebted to Chris Zink, whom I first met as a rotation advisor, but whose role in my career has grown into that of

a second mentor. It was she that suggested that the Dietz lab might be the best place to pursue my interests. She has also been a ferocious advocate for me, giving me the sage advice to never underestimate myself. It is her encouragement that gives me the strength to constantly try and go beyond my limits. I'd also like to thank Jonathan Butcher and his student Russell Gould, who collaborated with me as a graduate student and has now joined the Dietz lab as a postdoc. I also thank David Huso and his technician Guosheng Liu, who helped with the animal surgeries. I will always remember David Huso's constant willingness to help in any way possible, and his sage advice.

In closing, I'd like to thank Leslie Lichter, Colleen Graham, and Rajini Rao of the Graduate Program in Cellular and Molecular Medicine. They were the first to welcome me into Hopkins and ever since coming here, have always been there to help me through. To all those who have made this possible, I thank you and hope to make you proud in the future.

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Chapter 1

Introduction

Fibrosis is a pathologic process characterized by increased extracellular matrix protein synthesis and deposition, resulting in the accumulation of scar tissue that progressively compromises physiologic organ function. Fibrotic diseases are currently a leading cause of mortality in the world and are often associated with a poor prognosis due to limited treatment options (Coultas, Zumwalt et al. 1994, Perez, Rogers et al. 2003). The etiologies of common fibrotic diseases such as autoimmune scleroderma, idiopathic pulmonary fibrosis, liver cirrhosis, and kidney fibrosis remain poorly understood, thus hindering the development of effective therapies. The current standard of care for patients with scleroderma involves suppression of the immune system and management of symptoms, such as constriction of blood flow (Raynaud's phenomenon) and joint/chest pain. These interventions, however, fail to halt or reverse pernicious organ fibrosis and, most importantly, fail to ameliorate the progressive pulmonary fibrosis most often associated with mortality. A comprehensive therapy for these fibrotic diseases must halt the progression of and/or reverse organ fibrosis in order to significantly improve patient outcomes.

In the past decade, numerous studies have linked increased calpain expression and/or activity to the development of tissue fibrosis, but an underlying molecular mechanism has yet to be defined. Calpains are Ca^{2+}-dependent non-lysosomal cysteine proteases that specifically cleave their target substrate based

on protein tertiary structure, usually leaving an intact functional domain that shows an alteration and often gain of activity upon the calpain-induced post-translational modification (Goll, Thompson et al. 2003). Calpain-mediated proteolysis is responsible for a wide array of functions within the cell, including remodeling of the cytoskeleton during cell migration (Dourdin, Bhatt et al. 2001)), cell cycle progression (Janossy, Ubezio et al. 2004), and apoptosis (Momeni 2011).

Suppression of calpain activity in mouse models has been shown to ameliorate thioacetamine-induced liver fibrosis (Wang, Chen et al. 2004), cardiovascular fibrosis after angiotensin II-infusion (Letavernier, Perez et al. 2008), bleomycin-induced pulmonary fibrosis (Tabata, Tabata et al. 2010)), hypoxia/monocrotaline-induced lung fibrosis (Ma, Han et al. 2011), and scar formation during wound healing (Nassar, Letavernier et al. 2012). In addition, studies have correlated calpain expression with cancer metastasis and poor outcome in patients with a variety of tumors of epithelial origin (Storr, Lee et al. 2012, Storr, Zaitoun et al. 2012, Storr, Pu et al. 2013). An in-depth understanding of the underlying mechanism behind this phenomenon has the potential to significantly contribute to the development of therapeutic agents against a pervasive pathologic mechanism that is relevant to diverse and common human disease phenotypes.

We hypothesized that the role that calpain activity plays in both fibrosis and in the development of epithelial tumor metastasis could be explained by its involvement in a cellular process known as Epithelial-to-Mesenchymal Transition

(EMT). While there is controversy (and likely heterogeneity) regarding the cell type of origin, the concept that most if not all fibrotic disease requires Transforming Growth Factor β (TGFβ)-induced mesenchymal transition into collagen-expressing myofibroblasts has been widely accepted (Leask and Abraham 2004, Wynn 2007). Likewise, mesenchymal transition of epithelial tumors is thought to be a prerequisite for local invasion and ultimate dissemination of cancer cells, with a prominent contribution from enhanced expression and activity of matrix metalloproteinases (MMPs) (Radisky and Radisky 2010, Nistico, Bissell et al. 2012). In breast cancer, stromal cells synthesize several isoforms of MMPs, but the tumor cells produce the majority of MMP-9, whose expression was found to promote metastasis in a mouse orthotopic model (Mehner, Hockla et al. 2014). An emerging model suggests that cancer cells can undergo EMT to become cancer stem cells (Mani, Guo et al. 2008, Shuang, Wu et al. 2014), gaining the ability for self-renewal and ability to resist chemotherapy (Pattabiraman and Weinberg 2014). Therapies designed to specifically target cancer stem cells have focused on inhibition of focal adhesion kinase or phosphoinositide 3-kinase/mammalian target of rapamycin, and are currently in clinical trials (Hart, Novotny-Diermayr et al. 2013, Shapiro, Kolev et al. 2014, Kolev, Wright et al. 2015).

The calpain superfamily currently consists of 14 known isoforms in mammals that are either ubiquitous or show restricted temporal and/or spatial expression. Identification of subsequent isoforms was based on sequence homology to domain II of calpain (first half of protease domain) exceeding 20-

25% (Goll, Thompson et al. 2003). The overwhelming majority of research has focused on the ubiquitously expressed calpain 1 (CAPN1 a.k.a. μ-calpain), which requires micromolar Ca^{2+} concentrations for activity (determined in cell free systems), or calpain 2 (CAPN2 a.k.a. m-calpain), which requires millimolar levels of Ca^{2+}. These high concentrations of Ca^{2+} are far above those present in a cell, which has led to several hypotheses to explain how the requirement level is either lowered or achieved, which remain unproven. CAPN1 and CAPN2 must heterodimerize with a small regulatory subunit, such as the ubiquitously expressed calpain small subunit 1 (CAPNS1 a.k.a. CAPN4), in order activate their catalytic protease activity. CAPN9 is the only other isoform that has been experimentally confirmed to heterodimerize with CAPNS1 (Ravulapalli, Campbell et al. 2009). Expression of CAPN9 is restricted to the gastrointestinal tract under physiological conditions (Hata, Abe et al. 2010)

A recently identified small subunit isoform, calpain small subunit 2 (CAPNS2), was experimentally proven to bind CAPN2 (Schad, Farkas et al. 2002), and described to be functionally similar to CAPNS1, inferring it can bind CAPN1 and CAPN9 as well. In contrast to the ubiquitously expressed CAPNS1, CAPNS2 showed highly restricted tissue-specific expression (Ma, Nakajima et al. 2004). The collective activity of these calpain heterodimers is regulated by the highly specific, potent and ubiquitously expressed endogenous calpain inhibitor calpastatin (Todd, Moore et al. 2003, Hanna, Campbell et al. 2008). Calpains cleave calpastatin – defining a mutually antagonistic relationship – but in most cases calpastatin activity dominates within the cell or tissue (Goll, Thompson et

al. 2003). The precise role of the activity of specific calpains has proven difficult to define with precision due to the existence of multiple isoforms and limitations of currently available inhibitors.

The characterization of various genetic knockout models has greatly added to our understanding of the biological role of this proteolytic system. *Capn1*$^{-/-}$ mice exhibited decreased platelet aggregation and diminished blood clot retraction, but this deficiency did not result in a clinically-relevant bleeding diathesis (Azam, Andrabi et al. 2001), a finding that is not attributable to redundancy by *Capn2* since it is not expressed in human platelets (Taylor, Christiansen et al. 1991). In contrast, *Capn2*$^{-/-}$ mice showed embryonic lethality, but a conditional knockout with preserved CAPN2 expression in the placenta has been shown to be viable suggesting an essential role in placental development and/or function (Takano, Mihira et al. 2011).

Homozygous, but not heterozygous, deletion of the *Capns1* gene in mice abrogated CAPN1 and CAPN2 protease activity in embryonic stem cells, and led to embryonic lethality due to impaired cardiovascular development (Arthur, Elce et al. 2000). *Capns1*$^{+/-}$ derived embryonic stem cells showed CAPN1 and CAPN2 protease activity indistinguishable from wild-type, and animals appeared phenotypically normal (Arthur, Elce et al. 2000). *Capn9*$^{-/-}$ mice displayed an increased susceptibility to ethanol-induced gastritis, but no other discernible phenotypes were observed (Hata, Abe et al. 2010).

Constitutive overexpression (under the control of a prion protein promoter) of the human calpastatin gene (*CAST*) in mice, resulted in decreased cleavage of

spectrin (a known calpain substrate) after brain injury, but no overt clinical phenotype (Schoch, von Reyn et al. 2013). The mild phenotype of these calpastatin-overexpressing mice is not surprising since during physiological conditions, calpastatin restrains but does not abrogate calpain activity (Goll, Thompson et al. 2003). Similarly, *Cast*$^{-/-}$ mice showed increased cleavage of spectrin in the hippocampus after injection of kainite (an inducer of excitotoxicity) and exhibited a decreased acoustic startle response, but were otherwise normal (Takano, Tomioka et al. 2005, Nakajima, Takao et al. 2008). The lack of a more overt phenotype could be explained by the redundant action of a similarly functioning calpastatin-like protein (such as high molecular weight calmodulin-binding protein (Kakkar, Raju et al. 1997, Singh, Shrivastav et al. 2008) and/or absence of a strong calpain-activating stimulus (such as ischemia-reperfusion injury).

In humans, three unrelated families were found to carry rare homozygous loss-of-function mutations in *CAST* that co-segregated with a disease phenotype characterized by skin blistering, dryness, scaling and peeling in association with, leukonchia (white discoloration of the nails), acral punctate keratosis (keratin buildup on body protrusions), angular cheilitis (inflammation of the corners of mouth), and knuckle pads (fibrous overgrowths over interphalangeal joints), collectively referred to as PLACK syndrome (Lin, Zhao et al. 2015). The disparity between the mild mouse and the severe human loss-of-function phenotypes appears difficult to reconcile, but might be explained by some residual calpastatin message, protein and activity in the targeted mouse line (Takano, Tomioka et al.

2005). Hyperactivity of calpains in humans has been associated with Alzheimer's disease (Nixon 1989), enhanced ischemia-reperfusion injury (Yoshida, Yamasaki et al. 1993), traumatic brain injury (Arrigoni and Cohadon 1991), cataract formation (Hightower, David et al. 1987) and cancer (Storr, Carragher et al. 2011). While calpain inhibitors have been proposed to treat these conditions (Carragher 2006, Bralic, Stemberga et al. 2012, Parameswaran and Sharma 2012), this class of inhibitors remains at the pre-clinical or early clinical stage of development.

This thesis establishes an obligate mechanistic connection between the activity of specific calpains and TGFβ-induced EMT and offers novel therapeutic strategies based on detailed characterization of this finding.

Chapter 2

Characterizing the Role of Calpain Proteases in Epithelial-to-Mesenchymal Transition

Introduction:

Epithelial-to-Mesenchymal Transition (EMT) is an inducible process that fundamentally alters cellular identity and behavior. EMT was first discovered as a process by which cell type diversity is established during gastrulation (Greenburg and Hay 1982, Hay 1982, Hay 1995). EMT that occurs in the context of normal embryo development has recently been categorized as Type I EMT (Kalluri and Weinberg 2009). EMT is also essential to drive the production of collagen-producing myofibroblasts in the wound healing process (Estes, Vande Berg et al. 1994) referred to as Type II EMT 2009 (Kalluri and Weinberg 2009). Finally, EMT appears critical to epithelial cancer metastasis in experimental systems (Ruiz and Gunthert 1996); while a direct human clinical correlate has yet to be found (Chui 2013), so-called type III EMT (Kalluri and Weinberg 2009) continues to be a major focus of cancer research (Ginnebaugh, Ahmad et al. 2014).

Epithelial cells are stationary by nature, tend to form large sheets with a cobblestone appearance in culture, are characterized by their strong cell-cell adhesions due to high E-cadherin expression, and have a cortical distribution of F-actin. In contrast, cells that have undergone EMT to become myofibroblasts lose their cell-cell adhesions due to loss of E-cadherin expression, are invasive/motile, form F-actin stress fibers, secrete large amounts of extracellular

matrix proteins/matrix degrading enzymes, and display spindle shaped morphology. Myofibroblasts are generally identified by their expression of alpha smooth muscle actin (αSMA), a mesenchymal protein marker.

Although several stimuli are known to induce EMT, most act through a limited number of overlapping pathways (Derynck, Muthusamy et al. 2014), which converge on activation of a group of master transcription factors (e.g. Snail, Slug, Zeb, and Twist) that repress expression of epithelial proteins or induce expression of mesenchymal proteins (Savagner, Yamada et al. 1997, Guaita, Puig et al. 2002, Zuniga, Quillet et al. 2002)

Hepatocyte growth factor was the first soluble protein found capable of inducing EMT (Stoker and Perryman 1985), a finding later generalized to other activators of receptor tyrosine kinases (Savagner, Yamada et al. 1997) including epithelial growth factor (Lu, Ghosh et al. 2003, Lo, Hsu et al. 2007, Zhang, Dong et al. 2014) and fibroblast growth factor (Valles, Boyer et al. 1990, Valles, Boyer et al. 1996, Billottet, Tuefferd et al. 2008). WNT signaling (Song, Zheng et al. 2015, Yang, Sun et al. 2015) and hedgehog signaling (Yamamichi, Shigemura et al. 2014, Ke, Caiping et al. 2015) have also been shown to activate EMT. Inhibition of WNT signaling using a monoclonal antibody (OMP-18R5) was able to suppress type III EMT in mice with human tumor xenografts, and is currently in clinical trials along with other similarly acting drugs (Gurney, Axelrod et al. 2012). TGFβ stands out as one of the most potent, widely applicable, and consistent inducers of EMT *in vitro*, with TGFβ1 most often implicated in cancer and/or fibrosis *in vivo* (Derynck, Muthusamy et al. 2014, Gonzalez and Medici 2014,

Zheng and Kang 2014). For this reason, early strategies aimed at EMT inhibition as a treatment for cancer or fibrosis naturally focused on TGFβ inhibition (Yingling, Blanchard et al. 2004, Denton, Merkel et al. 2007, Connolly, Freimuth et al. 2012). TGFβ ligands (types 1-3) are homodimeric molecules that are secreted from the cell in the context of a large latent complex (LLC) that includes a dimer of their processed N-terminal propetide (latency-associated peptide or LAP) and one of three latent TGFβ binding proteins (LTBPs 1, 3 or 4). The LLC is sequestered within the extracellular matrix by virtue of direct biochemical interactions between LTBPs and structural matrix proteins (e.g. fibronectin or fibrillins). Latent TGFβ is converted into active TGFβ through a variety of mechanisms. Free TGFβ ligands bind to the type II TGFβ receptor subunit (TβRII), promoting the recruitment and activation (via phosphorylation) of the type I TGFβ receptor subunit (TβRI, a.k.a. ALK5) within the GS domain, resulting in a conformational change that opens up the kinase domain. The activated type I TGFβ receptor subunit then binds and phosphorylates receptor-activated SMADs (SMAD2 or SMAD3), which then partner with SMAD4, translocate to the nucleus and mediate transcriptional responses (in partnership with other transcription factors).

Attempts have been made to inhibit the TGFβ pathway through targeting activators (e.g. αv integrins), ligand-receptor interactions (e.g. using neutralizing antibodies) or TGFβ receptor kinase activity (e.g. using small molecules). There has been concern that proximal inhibition may be vulnerable to pathologic workaround strategies (i.e. due to redundancy or compensation) and to side

effects that relate to loss of the pleiotropic physiologic (and beneficial) functions of TGFβ, prominently including wound healing, suppression of inflammation, tumor suppression, coordination of adaptive immunity and tissue morphogenesis. In light of these limitations, we have chosen to focus on a strategy aimed at identification of critical distal events (and hence vulnerabilities) in TGFβ-induced EMT. In theory, distal inhibition would preferentially target pathologic but not physiologic functions of TGFβ signaling. In this chapter, we asked whether calpain activity is required for TGFβ-induced mesenchymal transition. If validated, calpains would emerge as attractive targets for inhibition of pathological EMT.

Methods:

Cell Culture

Namru Mouse Mammary Gland Epithelial Cells (NMuMG) were obtained from American Type Culture Collection (CRL-1636). Cells were cultured and maintained in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 10μg/mL insulin. Recombinant TGFβ1 was purchased from R&D Systems (240-B-010) and reconstituted in sterile water with 1mg/mL bovine serum albumin (BSA) and 4mM sterile cell culture grade hydrochloric acid purchased from Sigma (H9892). To induce EMT, NMuMG cells were first starved and pretreated with pharmacological inhibitors in 0.5% serum DMEM for 24 hours, prior to treatment with TGFβ1 (5ng/mL).

Small Molecule Inhibitors

MDL-28170 was purchased from Enzo Lifesciences (BML-PI130) and dissolved in DMSO for a 100mM stock solution. Calpeptin was purchased from Tocris (0448) and dissolved in DMSO for a 100mM stock solution. 2-APB was purchased from Sigma (D9754) and dissolved in DMSO for a 100mM stock solution. CA-074-OMe was purchased from Sigma (C5857) and dissolved in DMSO for a 100mM stock solution. SB431542 was purchased from Sigma (S4317) and dissolved in DMSO for a 10mM stock solution.

Western Blot

Cells were harvested from dishes/plates using radio immunoprecipitation assay buffer (RIPA) and sonicated. Protein concentration for samples was quantified by colormetric BCA Assay from Pierce (23225). Protein samples were prepared with Bio-Rad XT reducing agent (161-0792) and Bio-Rad XT sample buffer (161-0791), which was denatured at 95^{o}C for 10 minutes. Samples were loaded onto Bio-Rad 10% Bis-Tris gels and separated by SDS-PAGE with MOPS buffer using the Bio-Rad Criterion system. Size separated proteins were transferred onto a methanol-activated Immobilon-FL polyvinylidene fluoride membrane from Millipore (IPFL00010) in 1x NuPage transfer buffer from Invitrogen (NP0006) with 10% methanol using the Bio-Rad Criterion Blotter with plate electrodes (170-3872) at 100V for 1 hour at 4°C. After protein transfer, membranes were blocked in Li-Cor Odyssey blocking buffer (927-40000) diluted 1:1 with Phosphate Buffered Saline (PBS) for 1 hour with constant rocking at

room temperature. Membranes were probed with mouse anti-αSMA from R&D (Clone 1A4 MAB 1420) at 1:1000, goat anti-GAPDH from Santa Cruz Biotechnology (sc-20457) at 1:5000, mouse anti-phosphoSMAD2 from Millipore (Clone A5S 04-953) at 1:500, or rabbit anti-Filamin A-C-terminal fragment from Epitomics (2242-1) in diluted blocking buffer overnight with constant rocking at 4°C. Membranes were washed for 5 minutes three times in TBS-T (1xTris Buffered Saline with 0.1% Tween-20), and probed with appropriate Li-Cor Odyssey secondary antibodies conjugated to fluorophores, for 1 hour at room temperature with constant rocking. Membranes were washed for 5 minutes three times in TBS-T and scanned using a Li-Cor Odyssey Infrared Imaging System. Proteins levels were quantified by measuring integrated intensity for each sample/lane using Odyssey Application Software 3.0. Data were processed using Prism 5.0. Statistics were done on each blot by normalizing to levels present at 72 hours of TGFβ1 alone treatment and p-values calculated using Students t-test.

Immunofluorescence

NMuMG cells were plated and grown on sterilized glass coverslips. Cells were starved in 0.5% serum for 24 hours. Cells purposed for eventual treatment with MDL-28170 were pretreated with the drug, prior to treatment with TGFβ1. Coverslips were washed in PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, washed in PBS and blocked in immunofluorescence blocking buffer (5% Donkey Serum, 0.1% TritonX-100, in

PBS) for 1 hour at room temp. They were then were probed with mouse anti-E cadherin from BD Biosciences (610405) 1:500 in immunofluorescence blocking buffer overnight at 4°C. Coverslips were washed with PBS and probed with immunofluorescence blocking buffer containing Donkey Anti-Mouse Alexa 488 (1:500) from Molecular Probes (A-21202), rhodamine-phalloidin (1:500) from Molecular Probes (R415), and DAPI (1:25000) from Molecular Probes (D1306) for 1 hour at room temperature in the dark. After washing with PBS, they were mounted on slides with Prolong Gold mounting media from Molecular Probes (P10144). Images were obtained using a Zeiss LSM multiphoton confocal microscope with 10x and 20x objective lenses.

Quantitative PCR

RNA was harvested from cells using Qiagen RNeasy Kit (74106) with DNase digestion (79254). RNA was quantified by Nanodrop and used to make cDNA using a high capacity RNA to cDNA kit from Applied Biosystems (4387406). Quantitative PCR was done using TaqMan® probes for mouse *Col1A1* (ABI Mm00801555_g1), *MMP2* (ABI Mm00439498_m1), *MMP9* (Mm00442991_m1), and *GAPDH* (Mm99999915_g1) as a loading control Samples were run on a QuantStudio™ 7 Flex Real-Time PCR system. C_t values were corrected for loading and calculated using the $2^{\wedge-\Delta Ct}$ method. The average numerical value of the TGFβ1 only treated sample was normalized to 1 for each gene. P-values were calculated using Student's t-test.

Results and Discussion:

TGFβ1 induces EMT and increases calpain activity, events that are blocked by the TGFβ-receptor kinase inhibitor SB431542

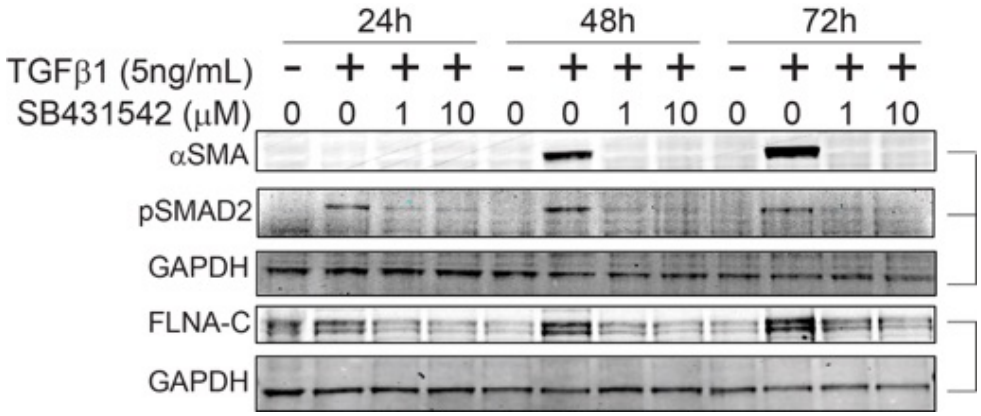
Different epithelial cell lines show a variable propensity for TGFβ-induced EMT *in vitro*, as defined by the extent of loss of epithelial markers and gain of mesenchymal ones (Brown, Aakre et al. 2004). We initially used the most robust and widely accepted cell line for this purpose: Namru mouse mammary gland (NMuMG) epithelial cells (Xie, Law et al. 2003, Brown, Aakre et al. 2004).

NMuMG cells treated with TGFβ1 underwent EMT, as evidenced by the progressive increase in expression of αSMA at 48 and 72 hours. As expected, this response was completely blocked upon administration of the TGFβ-receptor kinase inhibitor SB431542 (Figure 1). TGFβ1 treatment induced phosphorylation of SMAD2, an early event in the canonical (SMAD-dependent) TGFβ signaling cascade (Yang, Piek et al. 2003). Residual pSMAD2 induction was seen at 24, 48 and 72 hours after treatment with TGFβ1, but was blocked by SB431542 at all time points.

Treatment with TGFβ1 increased calpain proteolytic activity, as evidenced by increased cleavage of filamin A, a known calpain substrate (Gorlin, Yamin et al. 1990), which resulted in accumulation of the filamin A C-terminal cleavage fragment (FLNA-C; Figure 1). TGFβ1-induced filamin A cleavage was blocked by treatment with SB431542. Intriguing correlations between TGFβ and calpain activity exists in the literature (such as the fact that calpain-inhibition can

suppress TGFβ1-induced events like apoptosis (Gressner, Lahme et al. 1997, Ma, Han et al. 2011) and cell migration (Leloup, Mazeres et al. 2006). However, these data represent the first direct evidence for TGFβ induced calpain activation. They also document that in this robust culture-based assay, TGFβ-induced EMT temporally associates with increased calpain activity.

A)



B)

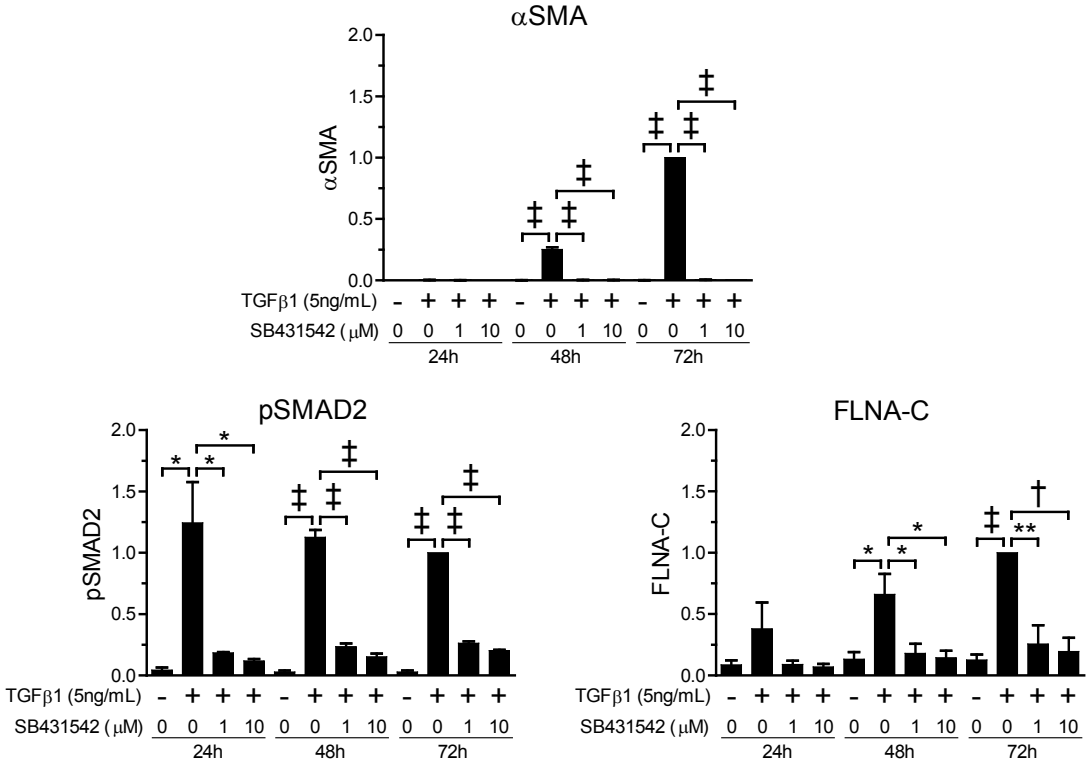


Figure 1: Western blot-assessment of markers of TGFβ1-induced EMT and calpain activity in the presence of TGFβ receptor kinase inhibitor SB431542. A) NMuMG (epithelial) cells were treated with TGFβ1 (5ng/mL) in the presence or absence of SB431542 (started 24 hours prior to stimulation with TGFβ1). B) Results are normalized to GAPDH expression and indexed to the 72 hour TGFβ1 alone signal (set to 1.0). Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001

Pharmacological calpain inhibitors suppress TGFβ1-induced EMT

MDL-28170 is a cell-permeable pharmacological calpain inhibitor within the peptide aldehyde class (Wang, Chen et al. 2004) and is frequently utilized for *in vitro* experiments. Like most pharmacological calpain inhibitors, it targets the protease active site (Lee, Morton et al. 2008).

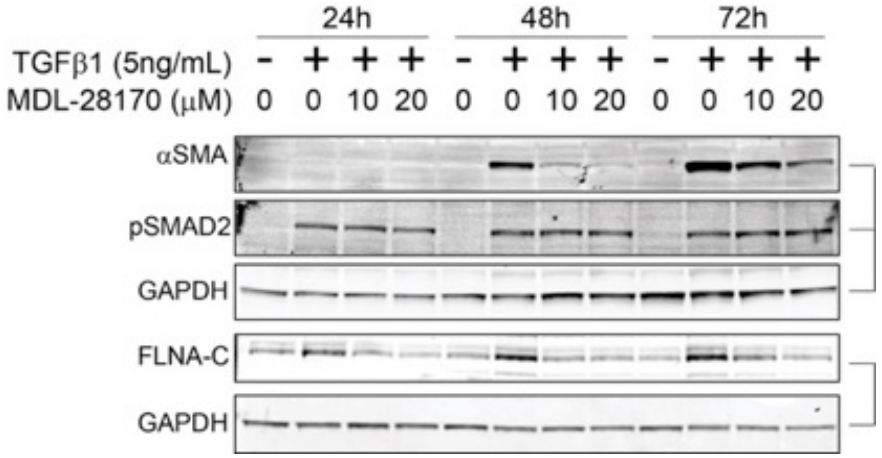
NMuMG cells treated with TGFβ1 and pharmacological calpain inhibitor MDL-28170 showed a dose-dependent inhibition of αSMA at 48 and 72 hours, relative to cells treated with TGFβ1 alone (Figure 2). Inhibition of calpain activity did not affect TGFβ1-induced phosphorylation of SMAD2, indicating that calpains are acting downstream of this event. The TGFβ1-mediated increase in calpain activity was prevented by the presence of MDL-28170, as seen by decreased accumulation of FLNA-C. These data provide the first indication that calpains played a critical role in EMT and that calpain inhibitors can block mesenchymal transition. These observations were also recapitulated using calpeptin (Tsujinaka, Kajiwara et al. 1988), another pharmacological calpain inhibitor (Figure 3).

Next we assessed the effect of pharmacological calpain inhibitors on the morphological changes associated with TGFβ1-induced EMT. NMuMG cells under steady state conditions express high levels of E-cadherin (an epithelial marker), localized at the cell membrane and concentrated at areas of cell-cell contacts (Figure 4). These cells also exhibited a cortical actin cytoskeleton arrangement typical of epithelial cells (Wu, Gomez et al. 2014). Treatment with TGFβ1 for 48 hours resulted in a dramatic downregulation of E-cadherin

expression, a defining event in EMT. TGFβ-1 treatment also promoted the reorganization of cortical actin into stress fibers, which is indicative of a motile cellular phenotype (Tojkander, Gateva et al. 2012). Treatment with MDL-28170 was able to suppress TGFβ1 induced E-cadherin downregulation and stress fiber formation. Subtle changes suggestive of altered cell morphology (increased cell size and flattening) were only partially inhibited by the calpain inhibitor, an observation that might be attributed to incomplete inhibition of EMT or to the possibility that some but not all TGFβ1-induced cellular events are prevented by the indicated doses of MDL-28170.

We used quantitative PCR to measure mRNA levels corresponding to important constituents of the fibrotic synthetic repertoire of myofibroblasts. Treatment with TGFβ1 for 48 hours resulted in increased mRNA expression for Col1A1 (encoding Collagen I), *Vim* (encoding Vimentin), *Mmp2* (encoding MMP-2), and *Mmp9* (encoding MMP-9). Calpain inhibition using MDL-28170, prevented these transcriptional responses to TGFβ1 (Figure 5).

A)



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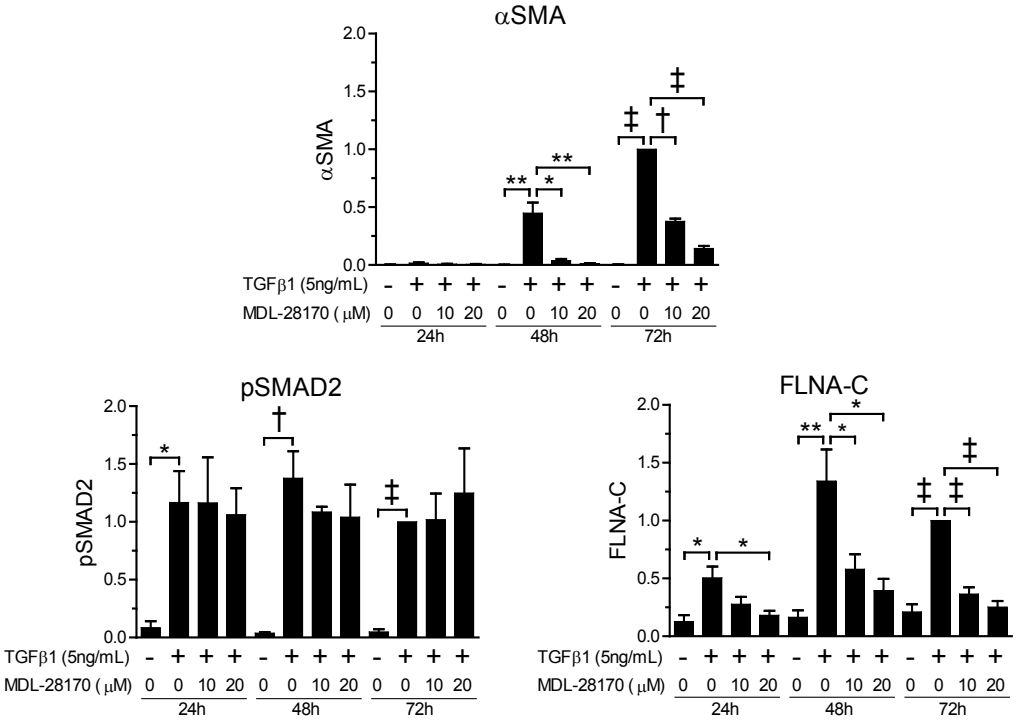
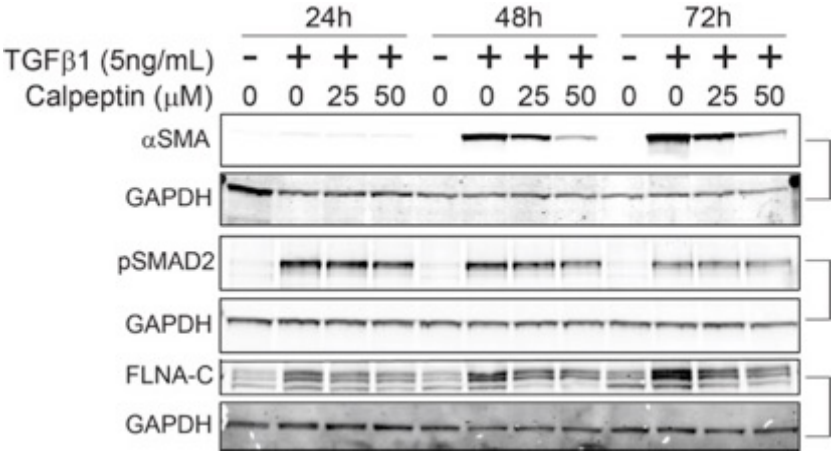


Figure 2: Western blot-assessment of markers of TGFβ1-induced EMT and calpain activity in the presence of the pharmacological calpain inhibitor MDL-28170. A) NMuMG (epithelial) cells were treated with TGFβ1 (5ng/mL) in the presence or absence of MDL-28170 (started 24 hours prior to stimulation with TGFβ1). B) Results are normalized to GAPDH expression and indexed to the 72 hour TGFβ1 signal (set to 1.0). Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001)

A)



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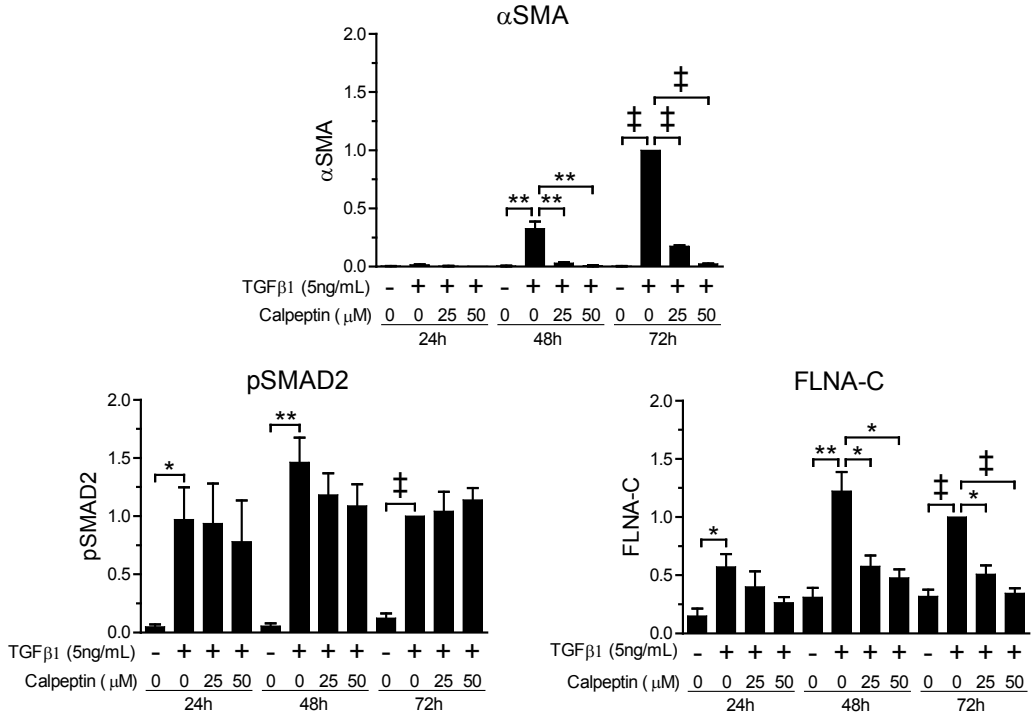


Figure 3: Western blot-assessment for markers of TGFβ1-induced EMT and calpain activity in the presence of pharmacological calpain inhibitor calpeptin. A) NMuMG (epithelial) cells were treated with TGFβ1 (5ng/mL) in the presence or absence of calpeptin (started 24 hours prior to stimulation with TGFβ1). B) Results are normalized to GAPDH expression and indexed to the 72 hour TGFβ1 alone signal (set to 1.0). Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

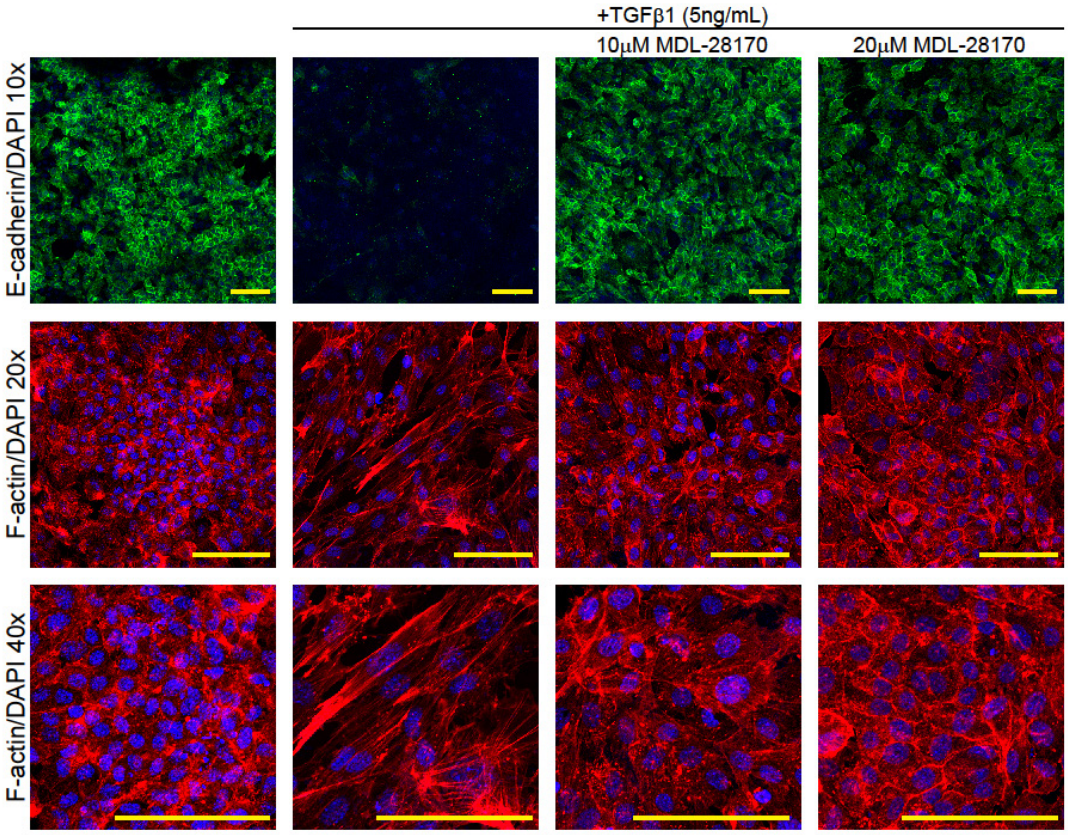


Figure 4: Treatment with pharmacological calpain inhibitor MDL-28170 inhibits morphological changes associated with TGFβ1-induced EMT. A) NMuMG (epithelial) cells were treated with TGFβ1 (5ng/mL) for 48 hours in the presence or absence of MDL-28170 (started 24 hours prior to stimulation with TGFβ1). Cells were stained for epithelial marker E-cadherin (Green), F-actin (red), and DAPI (blue) Scale bar: 100μm.

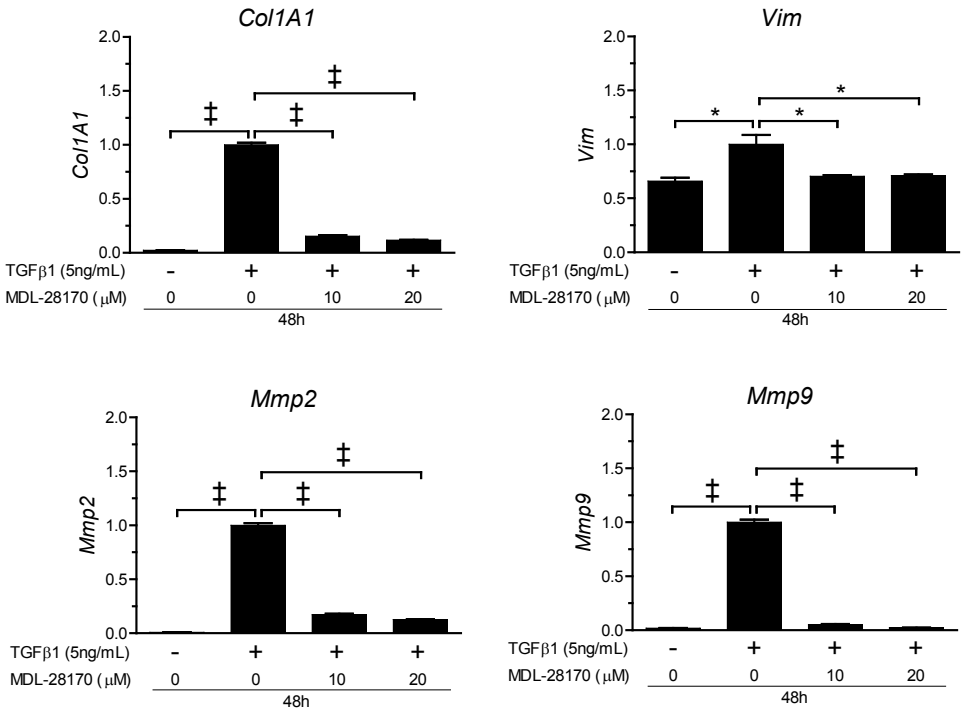
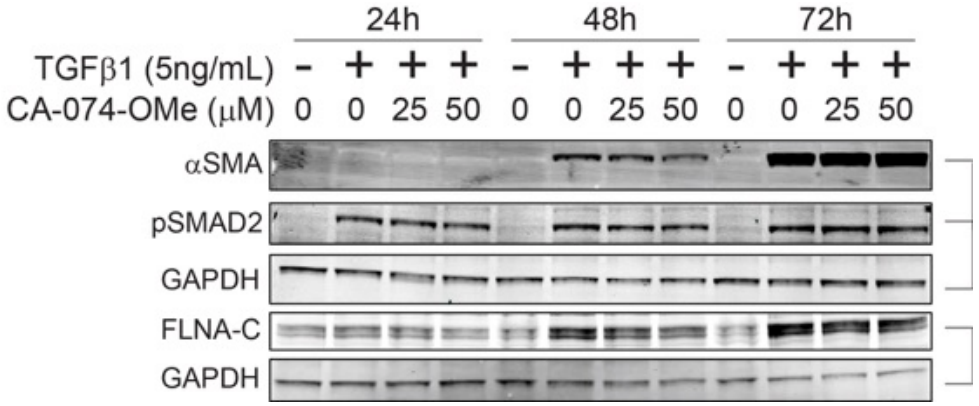


Figure 5: Expression of TGFβ1-induced EMT-associated gene products are suppressed in presence of pharmacological calpain inhibitor MDL-28170. NMuMG (epithelial) cells were treated with TGFβ1 (5ng/mL) for 48 hours in the presence or absence of MDL-28170 (started 24 hours prior to stimulation with TGFβ1). Results are normalized to *Gapdh* expression and indexed to levels in TGFβ1 alone treated samples. Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

Cathepsin B inhibitor CA-074OMe has no effect on TGFβ1-induced EMT

Small molecule calpain inhibitors are known to partially inhibit cathepsin B (Neffe and Abell 2005), a calcium-independent lysososomal cysteine protease involved in autophagy (Uchiyama 2001). To confirm that MDL-28170/calpeptin-mediated inhibition of EMT was selectively due to calpain-inhibition, we tested the effect of a methyl ester form of the specific cathepsin B inhibitor CA-074 (CA-074OMe) on EMT. This modified from was chosen because of its improved cellular permeability (Buttle, Murata et al. 1992). As seen in Figure 6, addition of CA-074OMe at doses previously demonstrated to be potently effective at inhibiting cathepsin B activity in culture systems (Reich, Wieczerzak et al. 2009, Matarrese, Ascione et al. 2010) did not influence TGFβ1-mediated SMAD2 phosphorylation, αSMA expression or filamin A cleavage. These data suggest that cathepsin B is not a critical effector of EMT, and that the EMT inhibition achieved by MDL-28170 or calpeptin was the result of calpain inhibition.

A)



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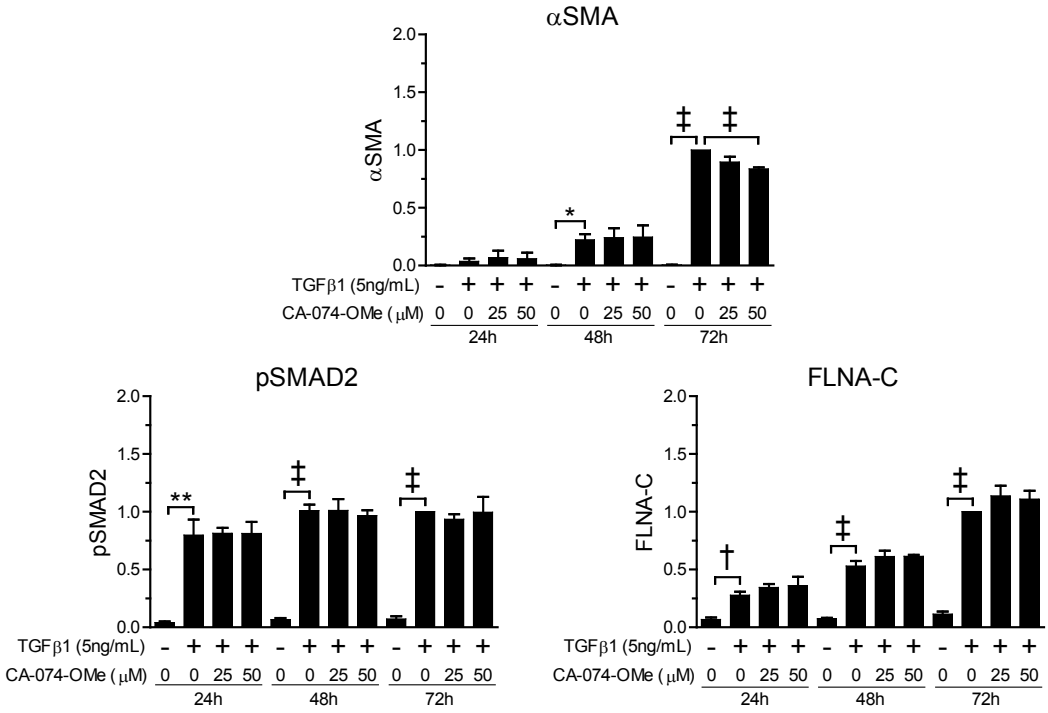


Figure 6: Western blot-assessment of markers of TGFβ1-induced EMT and calpain activity in the presence of cathepsin B inhibitor CA-074-OMe. A) NMuMG (epithelial) cells were treated with TGFβ1 (5ng/mL) in the presence or absence of CA-074-OMe (started 24 hours prior to stimulation with TGFβ1). B) Results are normalized to GAPDH expression and indexed to the 72 hour TGFβ1 alone signal (set to 1.0). Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

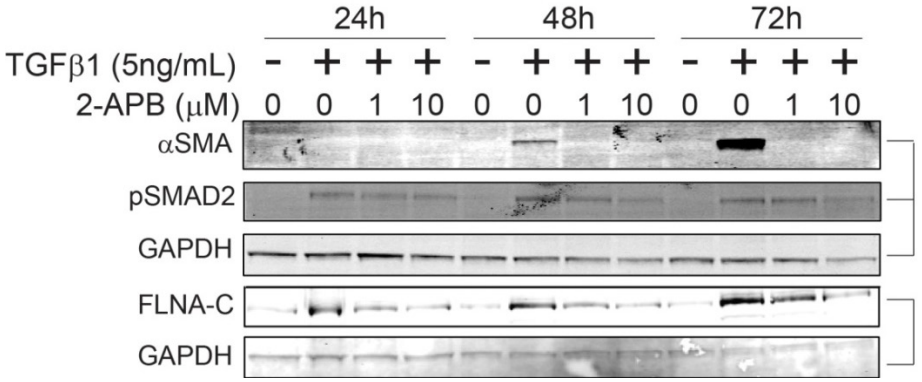
Ion-channel blocker 2-APB inhibits TGFβ1-induced EMT

The divalent cation channel TRPM7 has been reported to be particularly important in mediating the calcium influx required for activation of calpain activity, but the exact mechanism as to how this is achieved is unknown (Su, Agapito et al. 2006). One possibility is that calpains closely localize with specific calcium channels, such as TRPM7, and get exposed to the higher end of the calcium influx gradient. EMT induced by mechanical wounding/scratching, hypoxia or epidermal growth factor were shown to be mediated by transient increases in cytosolic calcium concentration in breast cancer cells (Davis, Azimi et al. 2014). This signal transduction pathway was found to be regulated by the expression of TRPM7. RNAi-mediated silencing of TRPM7, but not TRPV3, TRPC4, TRPC5, TRPC6, TRPM7, or ORAI1, prevented EGF-induction of vimentin expression in breast cancer cells (Davis, Azimi et al. 2014). Due to the delicate nature of cellular calcium homeostasis, global calpain manipulation via calcium chelators (EDTA) or calcium-ionophores (ionomycin) would be incompatible with long term experiments such as those required to assess TGFβ1-induced EMT. To assess for a calcium-flux dependent mechanism in TGFβ1-induced EMT (as would be predicted for a calpain-specific mechanism), we used 2-APB, a potent inhibitor of both IP_3-gated and TRP channels, including TRPM7 (Su, Agapito et al. 2006).

Treatment with 2-APB was able to block TGFβ1-induced expression of αSMA in NMuMG cells (Figure 7). 2-APB did not affect TGFβ1-induced phosphorylation of SMAD2, as was also seen with broad-spectrum calpain inhibitors. TGFβ1-induced calpain activity was suppressed in the presence of 2-

APB. These data suggest that EMT is sensitive to manipulations that restrict extracellular calcium influx and/or release of intracellular calcium stores, as expected for a calpain-dependent process.

A)



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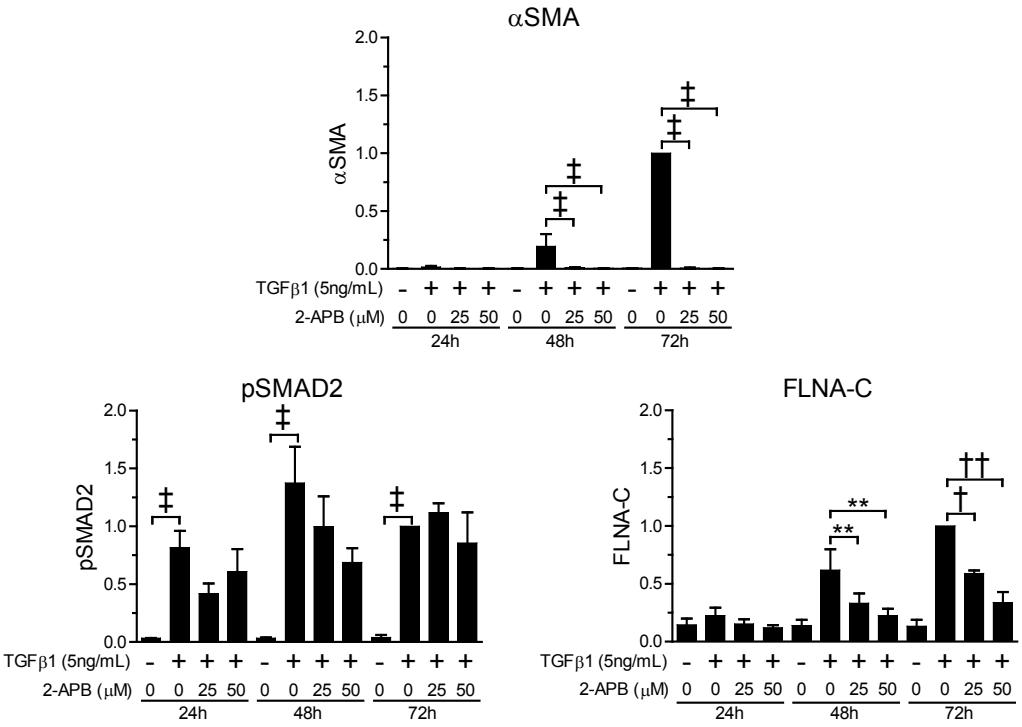
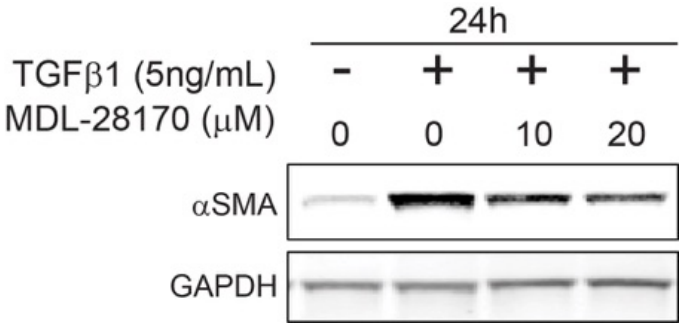


Figure 7: Western blot-assessment of markers of TGFβ1-induced EMT and calpain activity in the presence of ion-channel inhibitor 2-APB. A) NMuMG (epithelial) cells were treated with TGFβ1 (5ng/mL) in the presence or absence of 2-APB (started 24 hours prior to stimulation with TGFβ1). B) Results are normalized to GAPDH expression and indexed to the 72 hour TGFβ1 alone signal (set to 1.0). Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

Inhibition of calpain activity suppresses TGFβ1-induced Fibroblast-to-Myofibroblast Transition (FMT)

Resident fibroblasts under pro-fibrotic conditions have the capacity to transform into myofibroblasts and promote organ fibrosis (Kendall and Feghali-Bostwick 2014). Recent data from a kidney fibrosis model suggests that almost half of the myofibroblast population derives from proliferating resident fibroblasts (LeBleu, Taduri et al. 2013). Due to this potential additional source of myofibroblasts in promoting fibrosis, we tested the role of calpains during Fibroblast-to-Myofibroblast transition (FMT). Primary Normal Human Lung Fibroblasts (NHLF) were treated with TGFβ1 alone (Pegorier, Campbell et al. 2010), or in combination with the calpain inhibitor MDL-28170 for 24 hours. Treatment with TGFβ1 caused an increase in αSMA expression, which was inhibited in the presence of the calpain inhibitor MDL-28170 in a dose-dependent manner (Figure 8). These data suggest that calpain inhibition may be an applicable strategy for multiple cell types susceptible to mesenchymal transition, potentially making it a potent therapeutic strategy.

A)



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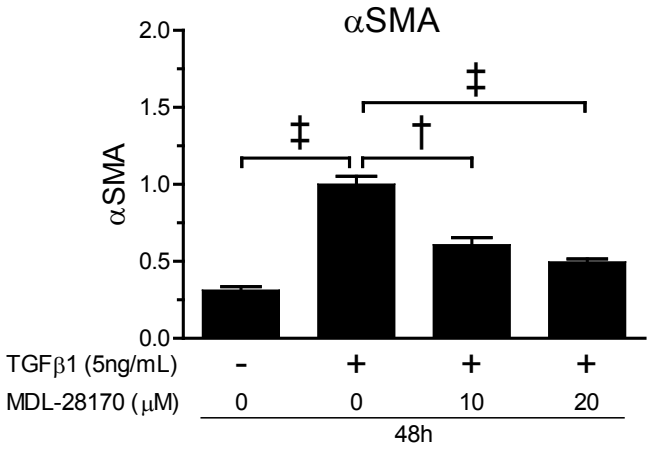


Figure 8: Western blot-assessment of a marker of TGFβ1-induced FMT in the presence of pharmacological calpain inhibitor MDL-28170. A) NHLF (lung fibroblast) cells were treated with TGFβ1 (5ng/mL) for 48 hours in the presence or absence of pretreatment with MDL-28170 (started 24 hours prior to stimulation with TGFβ1). B) Results are normalized to GAPDH expression and indexed to the TGFβ1 alone signal (set to 1.0). Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *$p<0.05$ **$p<0.01$ †$p<0.005$ ‡$p<0.001$.

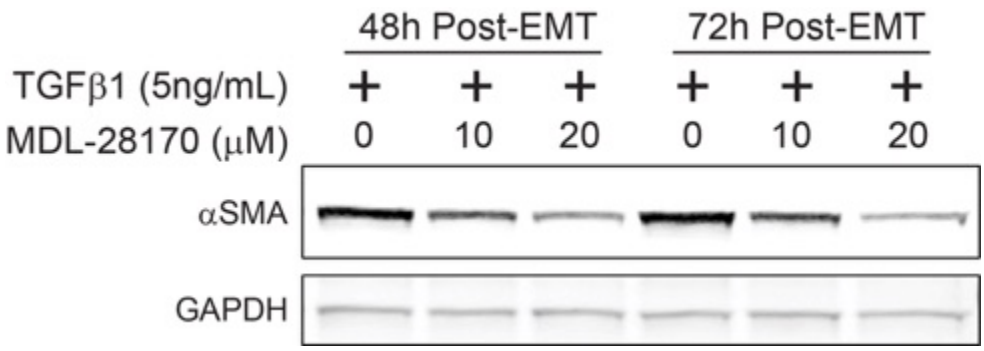
Suppression of calpain activity induces Mesenchymal-to-Epithelial Transition

Mesenchymal-to-Epithelial transition (MET) is a process in which a cell loses myofibroblast characteristics and adopts an epithelial phenotype. MET is involved in early development, best characterized in kidney organogenesis (Kreidberg, Sariola et al. 1993). MET has also been implicated in the ability of metastasizing cells to establish tumors at distant sites (Yang and Weinberg 2008) and potentially complicates the use of EMT-inhibitors in cancer therapy. In the context of fibrosis, MET could provide a highly desirable therapeutic benefit for patients in later stages of disease. We thus assessed the effect of inhibiting calpain activity in cells that had already undergone EMT. NMuMG cells were treated with TGFβ1 for 72 hours to induce EMT. Cells were then treated with MDL-28170, in addition to TGFβ1, for 48 and 72 hours (Figure 9). Cells treated with TGFβ1 and MDL-28170 showed a dose-dependent regression in αSMA expression over time, when compared to cells that received only TGFβ1.

The MET process requires not only loss of mesenchymal markers, but also reacquisition of an epithelial cell expression repertoire. We therefore characterized the effect of calpain inhibition on E-cadherin expression in cells that had undergone EMT. NMuMG Cells were grown to confluence on glass coverslips, serum starved (0.5% serum) for 24 hours, and treated with TGFβ1 for 72 hours to establish EMT (Figure 10A). We then sought to assess the effect of removing TGFβ from the media, or maintaining TGFβ1 with the addition of MDL-28170 or the TGFβ receptor kinase inhibitor SB431542. Cells were harvested after 48 or 72 hours and analyzed by immunofluorescence. Reacquisition of E-

cadherin expression was observed at both time points in cells that had TGFβ1 removed or were treated with a TGFβ receptor kinase inhibitor (Figure 10B,C), as previously reported (Gal, Sjoblom et al. 2008). We were able to demonstrate for the first time that the same effect is achieved upon calpain inhibition despite ongoing exposure of the cells to TGFβ1 (Figure 10B,C). These data suggest that calpain activity is required to both induce and maintain a mesenchymal state in response to TGFβ.

A)



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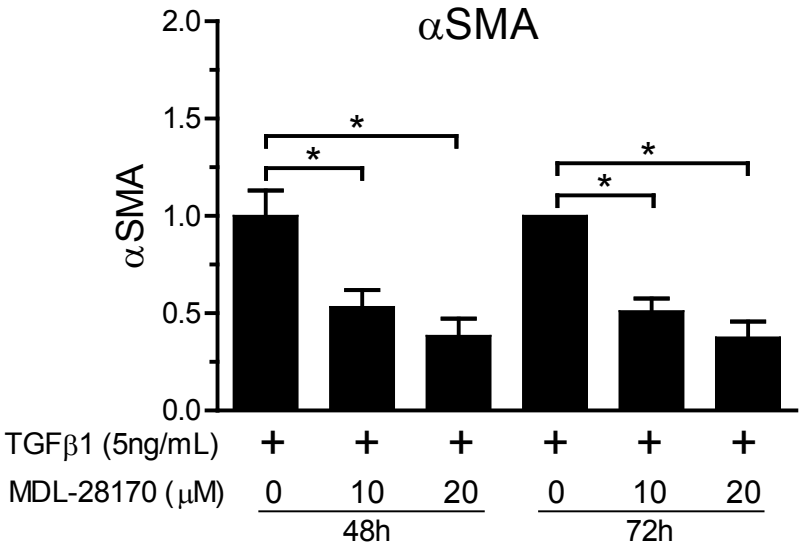


Figure 9: Pharmacological calpain inhibitor MDL-28170 induces Mesenchymal-to-Eplithelial transition. A) Representative western blot of NMuMG cells pre-treated for 48 hours with TGFβ1 followed by treatment with TGFβ1±MDL-28170 for 48 or 72 hours. Membranes probed for αSMA and GAPDH as a loading control. B) Results are normalized to GAPDH expression and indexed to the 72 hour post-EMT TGFβ1alone signal (set to 1.0) Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.001 †p<0.005 ‡p<0.001.

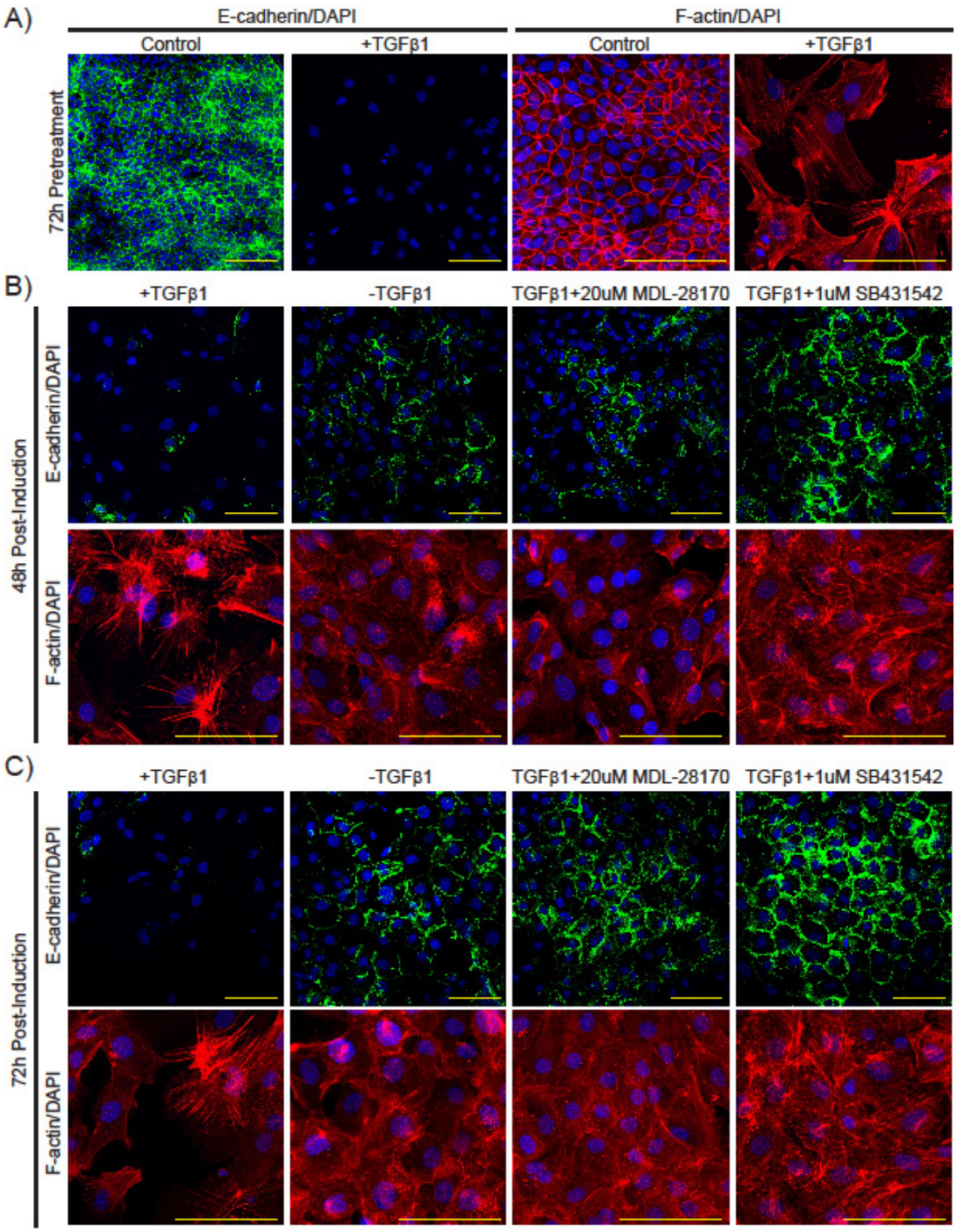


Figure 10: Treatment with pharmacological calpain inhibitor MDL-28170 induces morphological changes associated with MET. A) NMuMG cells were treated with TGFβ1 (5ng/mL) for 72 hours to establish EMT and verified by loss of E-cadherin and formation of stress fibers. These cells were then maintained in media with TGFβ1, media that was not supplemented with TGFβ1, media containing TGFβ1 and MDL-28170, or media with TGFβ1 and TGFβ receptor kinase inhibitor SB431542 for B) 48 hours C) 72 hours. Cells were stained for the epithelial marker E-cadherin, and F-actin. Scale bar: 100μm.

Chapter 3

Calpastatin-Mediated Inhibition of Epithelial- and Endothelial-to-Mesenchymal Transition Implicates Dimeric Calpain Isoforms

Introduction:

Calpain activity is physiologically regulated by the expression of calpastatin, an endogenous calpain inhibitor that prevents catastrophic unregulated proteolysis inside the cell (Goll, Thompson et al. 2003). Calpastatin is the most specific calpain inhibitor known with an IC_{50} in the low nanomolar range (Hanna, Campbell et al. 2008), likely the result of co-evolution with the enzyme (Betts, Weinsheimer et al. 2003, Tompa 2010).

Global calpain activity within the cell is determined by the relative levels of calpastatin expression and calpain proteolytic activity. Under typical physiological conditions, calpastatin reversibly binds and suppresses calpain activity (Goll, Thompson et al. 2003). Sufficiently high calpain activity can overcome this suppression by cleaving calpastatin, suggesting a mutually antagonistic relationship.

Calpastatin exclusively inhibits dimeric calpains because it requires binding to both the large catalytic subunit (CAPN1, CAPN2, CAPN9) and the small regulatory subunit (CAPNS1, CAPNS2) (Hanna, Campbell et al. 2008). Delineation of the structure of the calpain-calpastatin complex revealed that calpastatin binds to regions flanking the calpain substrate-recognition cleft, but

loops out of the protease active site to escape cleavage (Hanna 2008). Calpastatin has high affinity for activated calpains due its recognition of supplementary binding sites that are present only in the Ca^{2+} bound conformation (Hanna, Campbell et al. 2008). Each calpastatin molecule contains four calpastatin inhibitory domains; while all domains are capable of inhibiting calpain, they have significantly different dissociation constants (Hanna, Campbell et al. 2008). The functional unit for calpain inhibition can be reduced to a 27 amino acid peptide encoded within Exon 1B (Ishima, Tamura et al. 1991, Betts, Weinsheimer et al. 2003)

Calpastatin has been studied most frequently with regard to its effect on degradation of post-mortem muscle proteins and commercial animal meat quality (Nonneman, Lindholm-Perry et al. 2011, Chung and Davis 2012, Bagatoli, Gasparino et al. 2013, Huang, Huang et al. 2014, Ropka-Molik, Bereta et al. 2014). With regard to EMT-associated metastasis, calpastatin expression was found to be significantly lower in tissue derived from invasive breast cancers compared to non-invasive breast tumors (Storr, Mohammed et al. 2011). In contrast, overexpression of calpastatin in melanoma cells of mice increased metastasis to lymph nodes and resistance to apoptosis, though the relevance of this artificial cancer model is likely limited (Raimbourg, Perez et al. 2013).

In experiments described in this chapter, we have over-expressed calpastatin in order to assess the effect of dimeric calpain activity inhibition on TGFβ-induced EMT and EnMT.

Methods:

Cloning (NMuMG Epithelial Cells)

A cDNA clone expressing full-length mouse calpastatin was purchased from Invitrogen (MGC 3710078). The coding sequence for calpastatin was amplified by PCR with primers containing restriction sites for EcoRI (5') and SalI (3'). Flanking restriction sites were digested with EcoRI and SalI to create sticky ends, and the cDNA amplimer was gel purified. The bicistronic vector pCMV-IRES-AcGFP from Clontech (632435) was digested with EcoRI and SalI, followed by treatment with antarctic phosphatase (NEB M0289) and the vector was gel purified. Vector and insert were ligated with a 1:3 ratio using T4 DNA ligase (M0202). Ligated DNA was used to transform OneShot TOP10 chemically competent cells from Invitrogen (C4040-10). Bacteria were plated on LB-ampicillin plates and positive clones screened by DNA digest and DNA sequencing.

Transfection (NMuMG Epithelial Cells)

NMuMG cells were transfected with pCMV-*Cast*-AcGFP or pCMV-IRES using Lipofectamine 2000 according to the manufacturer's instructions. Cells were transfected for 5 days, split, and selected for stably integrated clones using Geneticin (400ug/mL). A polyclonal cell line was established and confirmed to express AcGFP by live cell fluorescent microscopy.

Western Blot (NMuMG Epithelial Cells)

Western blot analysis was carried out using the same procedures outlined in Chapter 2 of this thesis unless otherwise indicated. Additionally, membranes were probed for AcGFP using Living Colors Av Monoclonal Antibody JL-8 from Clontech (632380) at a 1:1000 dilution.

Cell Culture (Endothelial Cells)

Primary Porcine Aortic Endothelial Cells (PAVEC) were harvested using the previously described methods (Gould and Butcher 2010). PAVECs were grown in flasks coated with 50 µg/mL rat tail collagen I from BD Biosciences (354249). Cells were cultured at 37^0C and 5% CO_2 in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 50 U/mL heparin. Culture medium was changed every 48 hours and cells were passaged with 0.05% Trypsin-EDTA 1:3 at confluence. The purity of endothelial population was monitored via quantitative real-time PCR, western blot, and immunofluorescent assessment. Only cultures with consistent *PECAM1* and *CDH5* (encoding VE-cadherin) expression, cobblestone morphology, and non-detectable *αSMA* expression were used. *αSMA* levels were measured via Quantitative PCR (> 37 cycle threshold), western blot, and immunofluorescence. PAVEC were used between passage four and six.

Transfection (Endothelial Cells)

PAVECs were electroporated in solution with plasmids encoding for either mouse calpastatin (pEF1α-*Cast*-IRES-IRFP) or empty vector (pEF1α-IRES-AcGFP) using the Neon transfection system (Invitrogen, Carlsbad, CA) as previously described (Mahler, 2013). Cells were then seeded and cultured for up to 48 hours before addition of TGFβ1 (10ng/mL) to induce EnMT. Cells were harvested at 48 hours after addition of TGFβ1.

RNA Extraction/Quantitative PCR (Endothelial Cells)

RNA extractions were performed using a Qiagen RNeasy Kit (74106) and RNA was reverse transcribed to cDNA using the Invitrogen SuperScript III RT-PCR kit with oligo(dT) primer (1808051-051). Sufficient quality RNA was determined by an absorbance ratio A260/A280 of 1.8-2.1, while the quantity of RNA was determined by measuring the absorbance at 260nm (A260). Quantitative PCR experiments were conducted using the Bio-Rad CFX96 cycler, with 40 cycles per sample. TaqMan® probes were used to detect expression of, *ACTA2* (encoding αSMA; Ss04245588_m1), *CDH1* (encoding E-cadherin, Ss03377287_u1), *VIM* (encoding Vimentin; Ss04330801_gH), *MMP2* (encoding MMP-2; Ss03394318_m1), *MMP9* (encoding MMP-9; Ss03392092_g1) and *GAPDH* (encoding GAPDH; Ss03375629_u1) as a loading control. C_t values were corrected for loading and calculated using the $2^{\wedge-\Delta Ct}$ method. The average numerical value of the TGFβ1 only treated sample was normalized to 1 for each gene.

Results and Discussion:

Overexpression of Calpastatin Suppresses TGFβ1-induced EMT

Overexpression of calpastatin remains one of the most specific methods to inhibit calpain activity. We designed a bicistronic vector containing cDNA for mouse calpastatin, followed by an internal ribosome entry site, and cDNA encoding Ac-green fluorescent protein. We chose this method to monitor calpastatin expression based on previous reports that calpastatin protein migrates unpredictably on SDS-PAGE gels (Takano, Maki et al. 1988), potentially confounding interpretation of western blots. The prevailing hypothesis is that calpastatin adopts an unstructured conformation in its unbound state, retarding movement in a gel. Calpastatin and empty vector control constructs were transfected into NMuMG cells and stable polyclonal cell lines were established.

Overexpression of calpastatin completely abrogated TGFβ1-induced αSMA expression in NMuMG cells when compared to controls (Figure 11). As expected, calpastatin expression also prevented TGFβ1-induced filamin A cleavage but did not influence proximal events in the canonical TGFβ signaling cascade (i.e. phosphorylation of SMAD2). TGFβ1-treated cells overexpressing calpastatin maintained expression of the epithelial marker E-cadherin and a cortical distribution of F-actin compared to TGFβ1 treated NMuMG cells transfected with an empty vector (Figure 12). The superior inhibition of TGFβ1-induced αSMA expression by calpastatin, when compared to the pharmacological inhibitors MDL28170 or calpeptin may relate to its greater

potency and specificity for calpain inhibition. However, it seems notable that calpastatin and pharmacologic inhibitors of calpains achieved comparable suppression of filamin A cleavage. The disparity between suppression of TGFβ1 induced αSMA and filamin A cleavage could be attributed to different relative contributions of various calpain isoforms to each event. As previously stated, calpastatin is known to inhibit CAPN1, CAPN2, and CAPN9. Among these calpain isoforms, CAPN9 is unique in its restricted expression pattern and in the amino acid sequence that characterizes its substrate binding cleft (compared to CAPN1 and CAPN2) (Davis, Walker et al. 2007). It follows that a competitive inhibitor, such as calpastatin, may have differential binding affinities for CAPN9 compared to CAPN1/CAPN2, although this hypothesis has not been specifically tested.

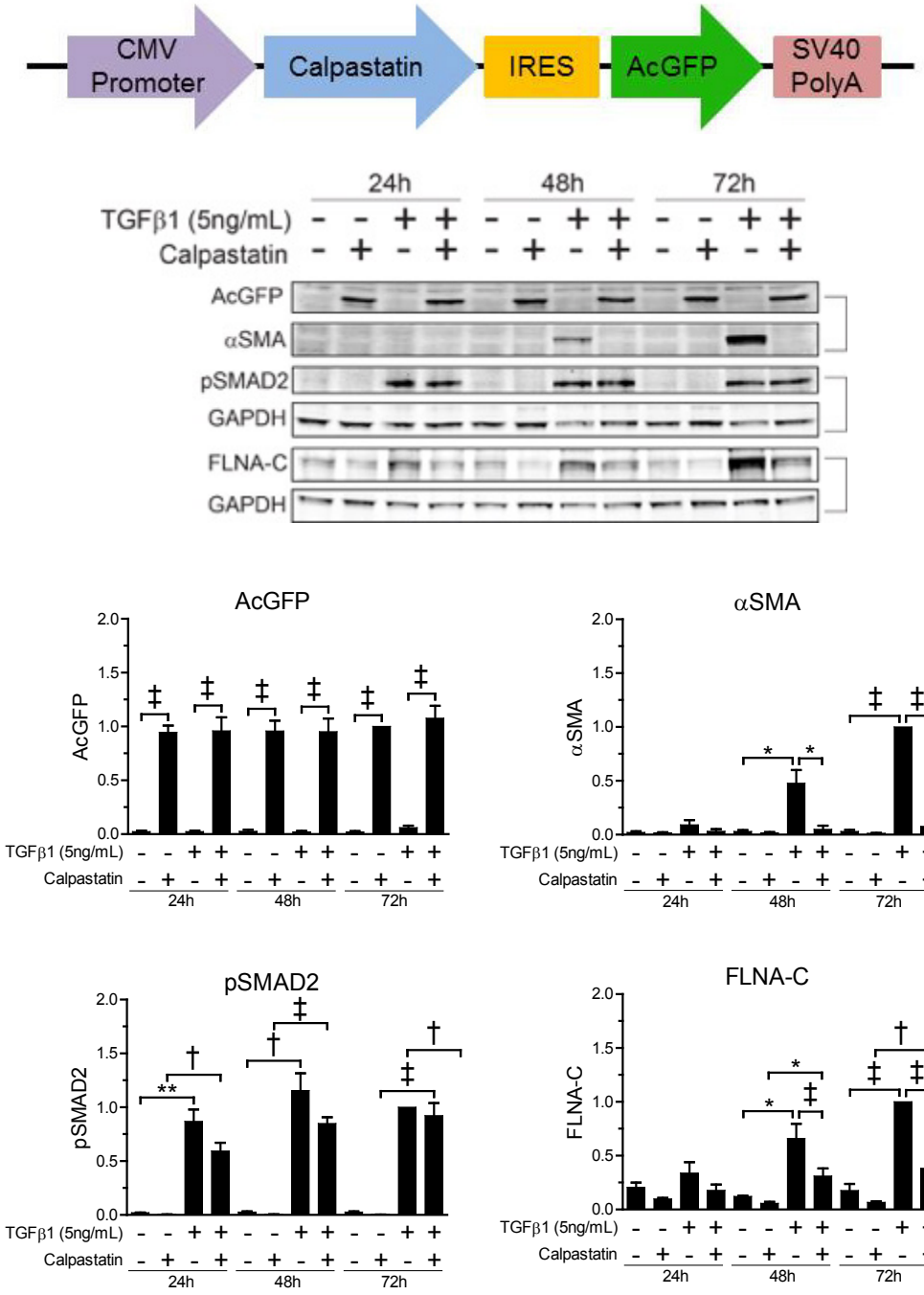


Figure 11: Western blot-assessment of markers of TGFβ1-induced EMT and calpain activity with overexpression of calpastatin. A) Stable cell lines derived from NMuMG cells transfected with calpastatin or an empty vector were treated with TGFβ1 (5ng/mL). B) Results are normalized to GAPDH expression and indexed to the 72 hour TGFβ1 alone signal (set to 1.0). Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

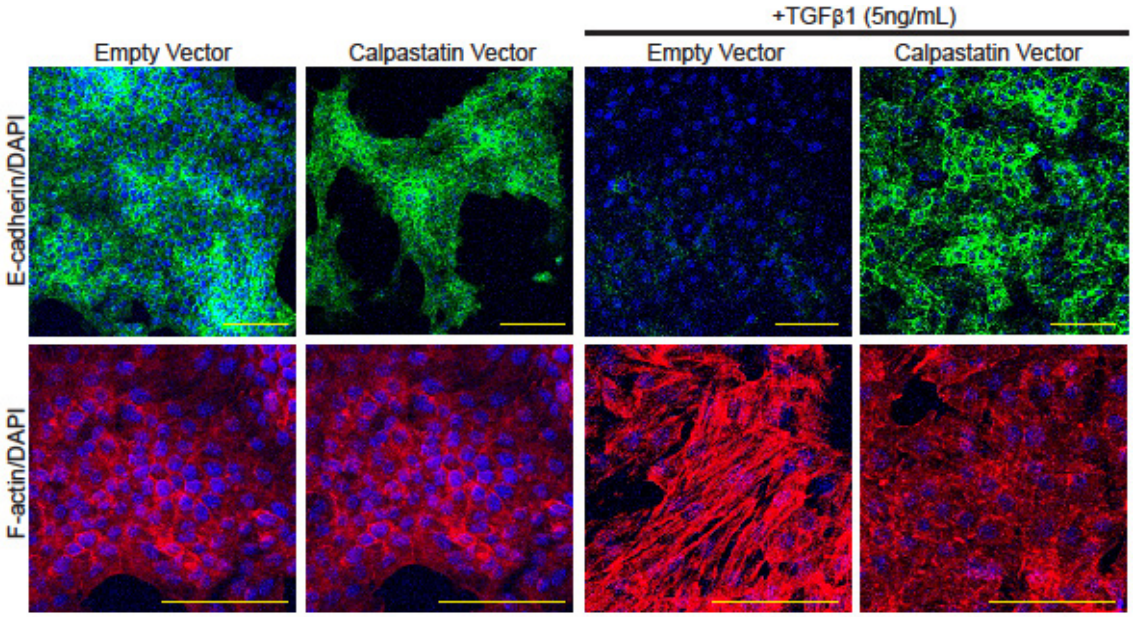


Figure 12: Overexpression of calpastatin inhibits TGFβ1-induced morphological changes associated with TGFβ1-induced EMT. Immunofluorescence NMuMG cells expressing calpastatin or empty vector after 48 hours of treatment with TGFβ1 (5ng/mL). Cells stained for epithelial marker E-cadherin (green), F-actin (red), and DAPI (Blue). Scale bar: 100μm.

Overexpression of Calpastatin Suppresses TGFβ1-induced EnMT

Endothelial-to-Mesenchymal Transition (EnMT) has been shown to play a pivotal role in pulmonary arterial hypertension (Arciniegas, Frid et al. 2007, Ranchoux, Antigny et al. 2015), chronic kidney disease associated-renal fibrosis (He, Xu et al. 2013), and cardiac fibrosis (Goumans, van Zonneveld et al. 2008, Yoshimatsu and Watabe 2011, Xu, Friehs et al. 2015). A previous study showed that suppression of calpain activity in bovine aortic endothelial cells abolished their ability to form stress fibers when plated on fibronectin (Kulkarni, Saido et al. 1999). Primary Porcine Aortic Valve Endothelial Cells (PAVEC) transfected with a control vector and treated with TGFβ1 for 48 hours expressed high levels of mRNA for *ACTA2* (encoding αSMA) and *VIM* (encoding vimentin), as measured by quantitative PCR (Figure 13). These cells also expressed high levels of mRNA for *MMP2/9* (encoding MMP-2 and MMP9, respectively) and low levels for *CDH1* (encoding E-cadherin), both indicators of EnMT. Transfection with a mouse calpastatin-expressing vector blocked TGFβ1-induced *ACTA2* expression and *CDH1* downregulation, but only partially suppressed *VIM* and *MMP2/9* mRNA expression. A prior study found that calpastatin-overexpressing transgenic mice exhibited decreased cardiovascular fibrosis after chronic angiotensin II infusion (Letavernier, Perez et al. 2008). Based on our data, this finding could have been due to suppression of EnMT. These data further support the hypothesis that proteolytic activity of a calpain heterodimer is required in multiple contexts for TGFβ1-induced mesenchymal transition.

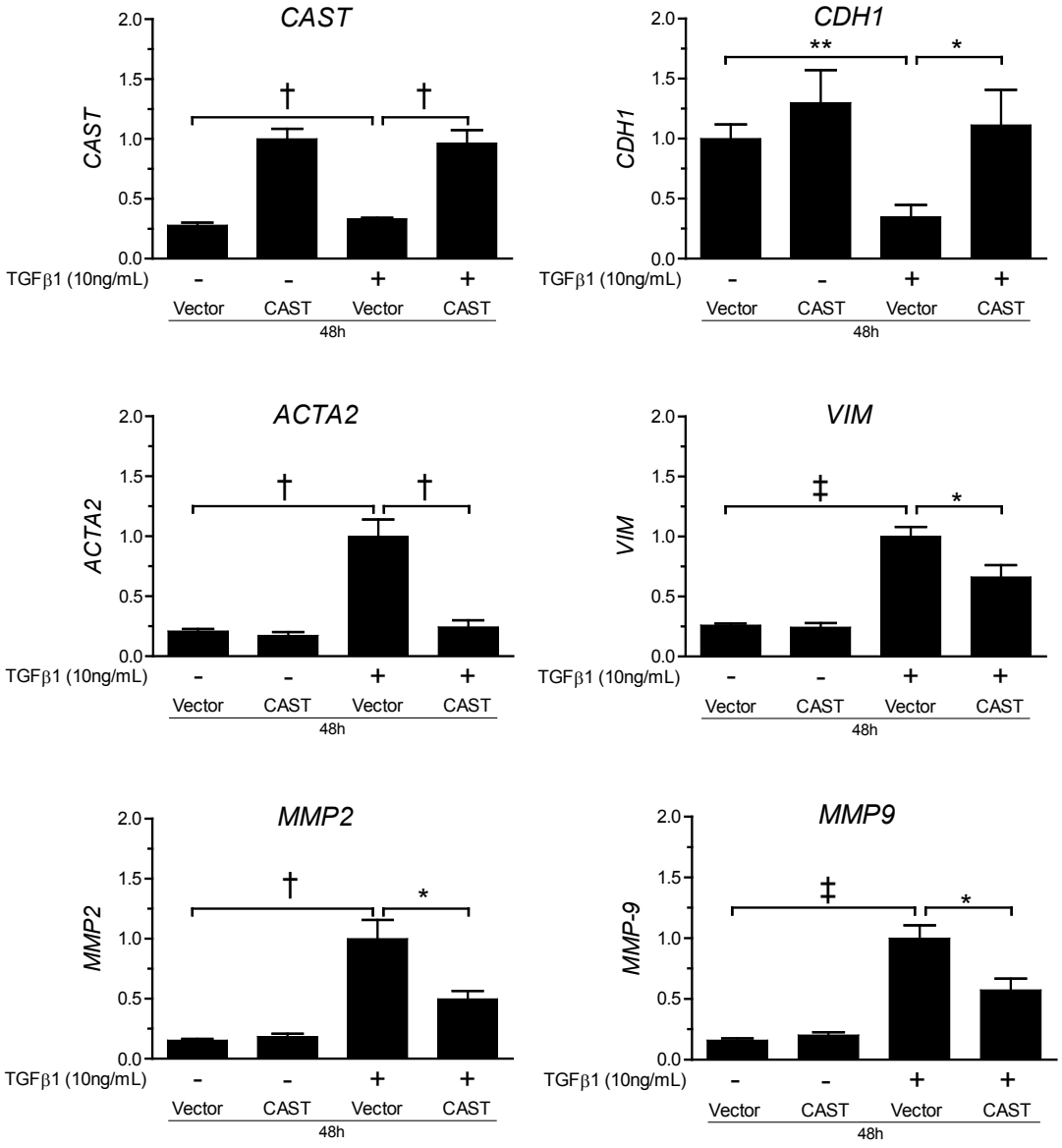


Figure 13: Quantitative PCR analysis of TGFβ1-induced expression of EnMT-genes in PAVEC (endothelial) cells overexpressing calpastatin. Cells were transiently transfected with a *Cast* expressing vector or empty vector for 48 hours, and then treated with TGFβ1 (10ng/mL) for an additional 48 hours. Messenger RNA expression levels were corrected for loading to *Gapdh* and normalized to the average value of samples transfected with control siRNA and treated with TGFβ1. Results reflect experiments performed in biological quadruplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

Chapter 4

The CAPN9/CAPNS2 Heterodimer is Critical for TGFβ1 Induced Epithelial- and Endothelial-to-Mesenchymal Transition

Introduction:

The data presented in the previous chapters strongly supported the hypothesis that the activity of a calpain heterodimers, which is inhibited by calpastatin, is a critical requirement for TGFβ1-induced mesenchymal transition in both EMT and EnMT. However, the exact molecular identity of this heterodimer remained to be elucidated. In cell-free systems, CAPN1 and CAPN2 are equally competent at cleaving most calpain substrates (Franco and Huttenlocher 2005). This suggests that isoform-specific functions at the cellular level are at least partly due to spatio-temporal regulation. Experimental strategies based on RNA interference (RNAi) have attempted to correlate individual calpain isoforms with specific cellular functions, but have often done so only within the context of a single cell-type. This can be problematic, especially in the case of highly similar calpains, such as CAPN1 and CAPN2, whose relative ratios differ between cell types in the same species or between same cell types in different species (Goll, Thompson et al. 2003).

A phylogenetic analysis of the calpain superfamily concluded that the *CAPN9* gene was present in a common vertebrate ancestor before the *CAPN1* and *CAPN2* genes diverged (Macqueen and Wilcox 2014). These data suggest

that *CAPN9* and *CAPN1*/*CAPN2* could have evolved in vertebrates to fulfill different functions. Structural analysis of the large catalytic subunits found that the amino acid residues lining the substrate recognition site in CAPN1 and CAPN2 were very similar to each other, but significantly different from analogous residues in CAPN9 (Davis, Walker et al. 2007). This structural difference could be the basis for differential substrate cleavage (Davis, Walker et al. 2007) and a unique function.

Current data regarding the recently discovered *CAPNS2* is limited. Human CAPNS1 and CAPNS2 show 63% amino acid identity with and an additional 16% of residues that are strongly similar (Schad, Farkas et al. 2002). CAPNS2 shares several functions with CAPNS1, such as the ability to heterodimerize with CAPN2 (Schad, Farkas et al. 2002), and it can be inferred the same is true for the other large subunits CAPN1 and CAPN9, although this has not yet been directly proven. In contrast, *Capns2* cannot compensate for the loss of *Capns1*, as evidenced by embryonic lethality of *Capns1*$^{-/-}$ mice (Arthur, Elce et al. 2000).

In Chapter 4, we have used an RNAi strategy to knockdown expression of individual dimeric calpain isoforms in order to reveal which are necessary for TGFβ1-induced EMT/EnMT.

Methods:

Transfection (NMuMG Epithelial Cells)

NMuMG cells were transfected with a SMARTpool mixture of 4 small interfering RNA (siRNA) purchased from GE Healthcare for each gene, targeting *Capn1* (M-062006-01-0010), *Capn2* (M-043027-01-0010), *Capns1* (M-048840-01-0010), *Capns2* (M-014858-00-0010) or non-targeting missense control (D-001206-13-50). Two siRNAs targeting *Capn9* were custom synthesized by GE Healthcare using previously validated sequences (Chen 2010). Cells were transfected using GE Healthcare Dhrmafect 4 (T-2004-03) according to the manufacturer's instructions. In brief, siRNA was diluted in Invitrogen OptiMEM I Reduced Serum Medium (11058021), as was Dharmafect 4. Diluted siRNA was mixed with diluted siRNA and incubated at room temperature for 20 minutes. NMuMG cells in DMEM containing 0.5% Serum and 10µg/mL insulin were treated with the siRNA/tranfection complex at a final siRNA concentration of 100nM.

Induction of EMT (NMuMG Epithelial Cells)

NMuMG cells were treated with TGFβ1 (5ng/mL) 24 hours after transfection with siRNA. Cells were treated with TGFβ1 for 48 hours as described in Chapter 2 of this thesis.

Western Blot

Western blot analysis was carried out using the same procedures outlined in Chapter 2 of this thesis with the following addendums. Membranes were probed with rabbit anti-CAPN1 from Cell Signaling Technologies (2556) at a 1:1000 dilution, rabbit anti-CAPN2 from Cell Signaling Technologies (2539) at a 1:1000 dilution, mouse anti-CAPN9 from Abnova (H00010753-M02) at a 1:1000 dilution, rabbit anti-CAPNS2 from Lifespan Biosciences (LS-C133503) at a 1:1000 dilution, in diluted blocking buffer overnight at constant rocking in 4C.

Cell Culture (Endothelial Cells)

Primary Porcine Aortic Endothelial Cells (PAVEC) were harvested and cultured as described in Chapter 2 of this thesis.

Transfection (Endothelial Cells)

PAVECs were electroporated in solution with siRNA targeting CAPNS2 from GE Healthcare (M-014858-00-0010) or non-targeting missense control from GE Healthcare (D-001206-13-50) using the Invitrogen Neon transfection system as previously described (Mahler, Farrar et al. 2013). Cells were then seeded and cultured for up to 48 hours before addition of TGFβ1 (10ng/mL) to induce EnMT.

RNA Extraction/Quantitative PCR (Endothelial Cells)

RNA extractions and quantitative PCR were conducted as described in Chapter 3.

Immunofluorescence (Endothelial Cells)

Samples were fixed in 4% paraformaldehyde overnight at 4C. Samples were then washed for 15 minutes on a rocker 3 times with PBS, permeabilized with 0.2% Triton-X 100 for 10 minutes, and washed another 3 times with PBS. Samples were incubated overnight at 4C in a 1% BSA blocking solution followed by another 4C overnight incubation with either rabbit anti-human E-cadherin from Abcam (ab53033) at a 1:100 dilution, and mouse anti-human vimentin from Invitrogen (18-0052) at a 1:100. After 3 washes for 15 minutes with PBS, samples were exposed to Alexa Fluor 488 or 568 conjugated, species-specific secondary antibodies at 1:100 in 1% BSA for 2 hours at room temperature. Three more washes with PBS for 15 minutes were followed by incubation with either DRAQ5 far red nuclear stain from Life Technologies (62251) at a 1:1000 dilution.

RNA Extraction/Quantitative PCR (Mouse Organs)

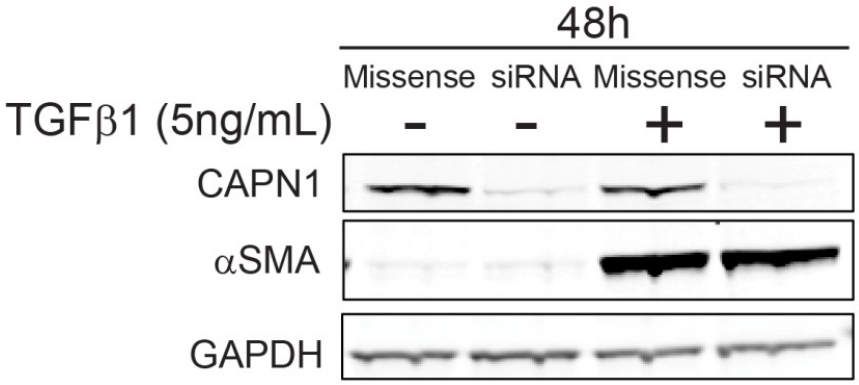
A C57Bl6 wild-type mouse was euthanized, perfused with PBS, and harvested for various organs. RNA extractions and quantitative PCR were conducted as described in Chapter 3. Quantitative PCR was performed using TaqMan® probes from Applied Biosystems for mouse *Capn1* (Mm00482964_m1), *Capn2* (Mm00486669_m1), *Capn9* (Mm00499260_m1), *Capns1* (Mm00501568_m1), *Capns2* (Mm03991531_s1), and *Gapdh* (Mm99999915_g1) as a loading control.

Results:

Knockdown of CAPN1 does not inhibit TGFβ1-induced EMT

CAPN1 expression was assessed by western blot and, as expected, it was found to be expressed in NMuMG cells under steady-state conditions; its levels did not change upon treatment with TGFβ1 (Figure 14). Knockdown of CAPN1 by siRNA had no effect on TGFβ1-induced expression of αSMA, suggesting that this isoform is not the critical driver of EMT. Loss of *Capn1* is seldom associated with severe phenotypic consequences, which may be due to *Capn2* upregulation and compensation.

A)



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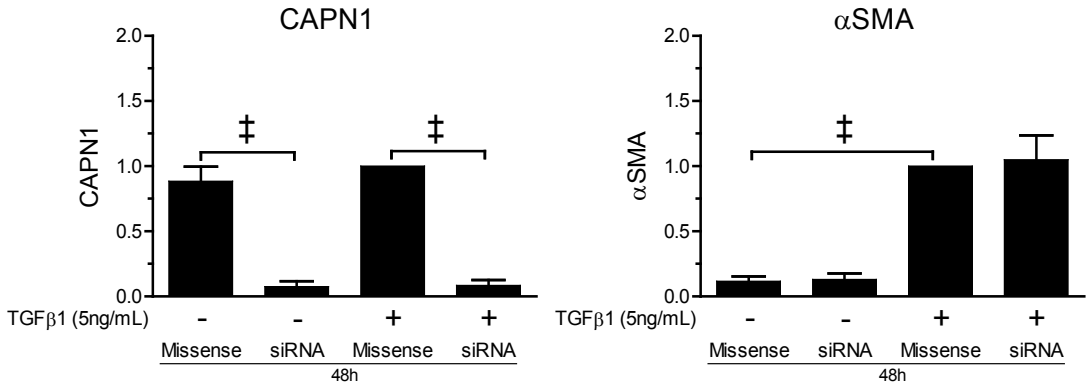


Figure 14: Western blot-assessment of a marker of TGFβ1-induced EMT after RNAi-mediated knockdown of *Capn1*. A) NMuMG (epithelial) cells were transfected with siRNA targeting *Capn1* or control siRNA 24 hours before treatment with TGFβ1 (5ng/mL). Cells were harvested 48 hours after treatment with TGFβ1 B) Protein levels were corrected for loading by GAPDH and normalized to the levels of present in samples transfected with a control siRNA and treated with TGFβ1. Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

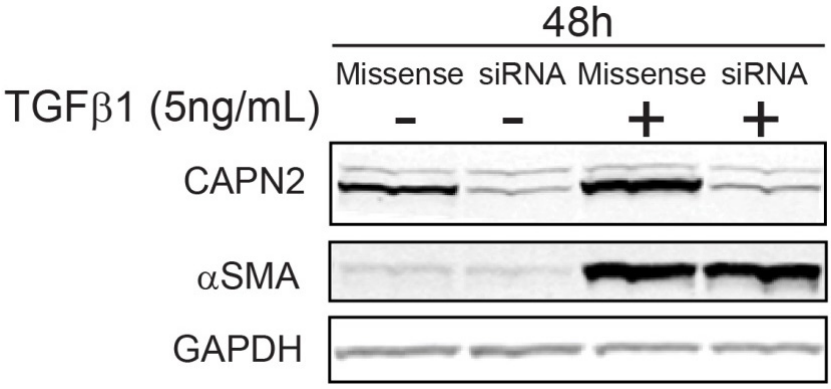
Knockdown of CAPN2 does not inhibit TGFβ1-induced EMT

CAPN2 has been shown to cleave several cytoskeleton-associated proteins (talin, spectrin, paxillin, cortactin, and focal adhesion kinase) (Franco, Perrin et al. 2004). Inhibition of CAPN2-mediated cortactin processing results in unregulated actin polymerization and protrusions in the plasma membrane (Perrin, Amann et al. 2006).

CAPN2 was expressed in NMuMG cells under steady-state conditions, but treatment with TGFβ1 increased its expression as assessed by western blot (Figure 15). Phorbol esters have also been shown to induce *CAPN2* expression in HeLa cells, but did not alter expression of *CAPN1* or *CAPNS1* (Hata, Ohno et al. 1992). Despite its induction, knockdown of CAPN2 expression did not affect TGFβ1-induced αSMA expression.

These data suggested that cleavage of structural proteins by CAPN2 is not required for EMT, and that perhaps cleavage of additional targets by other calpain isoforms is driving expression of EMT-associated genes.

A)



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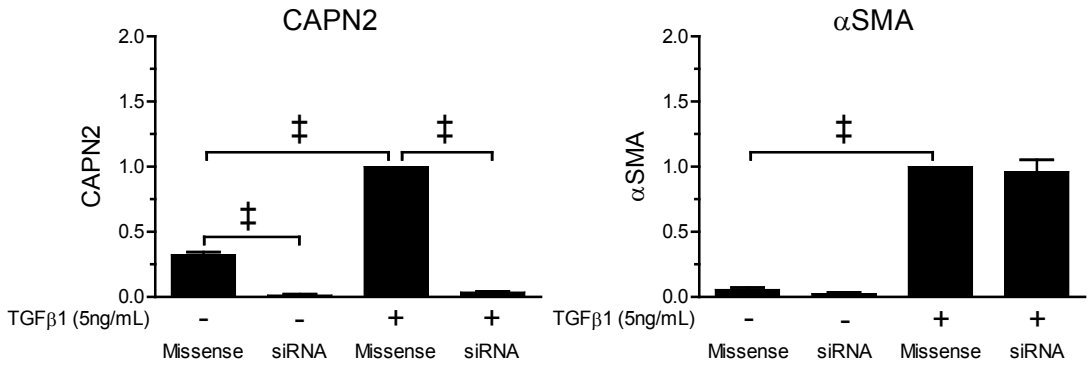
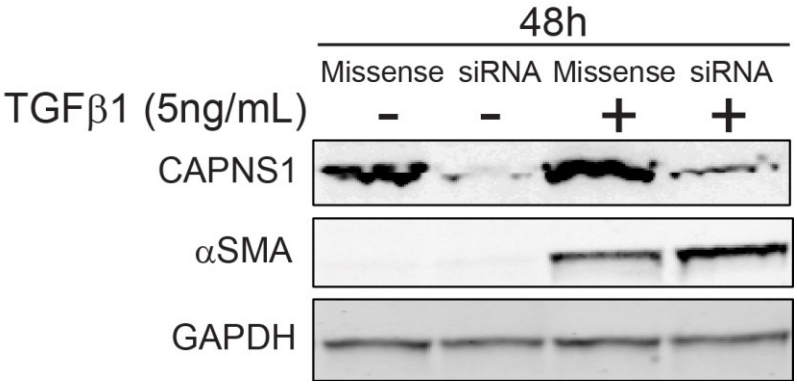


Figure 15: Western blot-assessment of a marker of TGFβ1-induced EMT after RNAi-mediated knockdown *Capn2*. NMuMG (epithelial) cells were transfected with siRNA targeting *Capn2* or control siRNA 24 hours before treatment with TGFβ1 (5ng/mL). Cells were harvested 48 hours after treatment with TGFβ1 B) Protein levels were corrected for loading by GAPDH and normalized to the levels of present in samples transfected with a control siRNA and treated with TGFβ1. Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

Knockdown of CAPNS1 does not inhibit TGFβ1-induced EMT

Knockdown of *CAPNS1* is a commonly used strategy to inhibit both CAPN1 and CAPN2. A rationale for adopting such an approach is to inhibit redundant activity between the two isoforms and achieve sufficient inhibition to affect function. CAPNS1 was expressed in NMuMG cells under steady-state conditions and was increased by treatment with TGFβ1. Despite this, knockdown of CAPNS1 by siRNA failed to inhibit TGFβ1-induced αSMA expression (Figure 16). These results corroborate the previous findings showing that knockdown of CAPN1 or CAPN2 had no effect on TGFβ1-induced EMT. This also excludes the possibility that the combination of CAPN1 and CAPN2 is required for TGFβ1-induced EMT. Until recently, CAPNS1 has been the only known small regulatory subunit component of a calpain heterodimer, making it necessary for all dimeric calpain function. The recent discovery of CAPNS2, however, has changed this paradigm. Dimeric calpain activity, found to be essential for TGFβ1-induced EMT (Figure 11) could still be mediated by CAPNS2 and CAPN9.

A)



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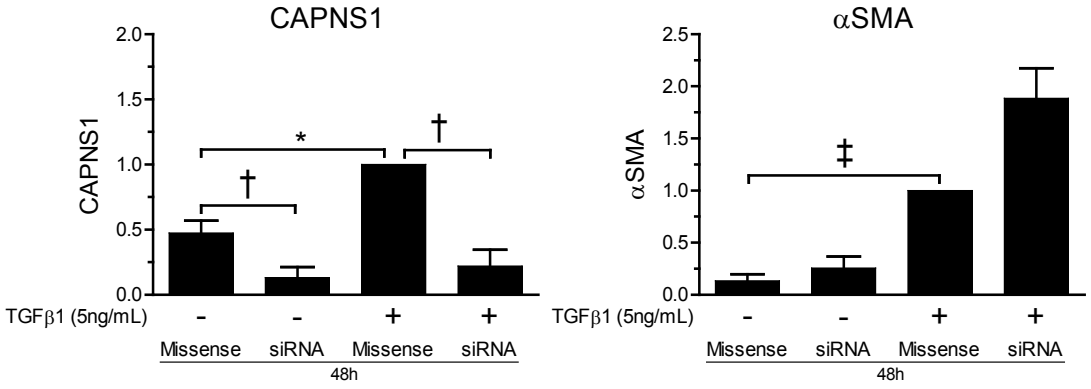


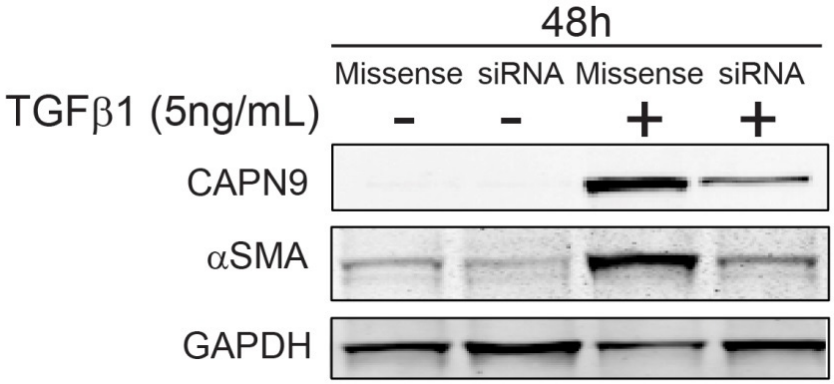
Figure 16: Western blot-assessment of a marker of TGFβ1-induced EMT after RNAi-mediated knockdown of *Capns1*. NMuMG (epithelial) cells were transfected with siRNA targeting *Capns1* or control siRNA 24 hours before treatment with TGFβ1 (5ng/mL). Cells were harvested 48 hours after treatment with TGFβ1. B) Protein levels were corrected for loading by GAPDH and normalized to the levels of present in samples transfected with a control siRNA and treated with TGFβ1. Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

Knockdown of CAPN9 inhibits TGFβ1-induced EMT

Recent experimental evidence has shown that CAPN9 can heterodimerize with CAPNS1 to form a functional enzyme (Ravulapalli, Campbell et al. 2009) and therefore could be a critical isoform mediating TGFβ1-induced EMT. However, under steady-state conditions, *CAPN9* expression is limited to the gastrointestinal tract (Hata, Abe et al. 2010). Western blot analysis showed that while CAPN9 is not expressed in NMuMG cells under steady state conditions, its expression is greatly increased by treatment with TGFβ1 (Figure 17). Importantly, partial knockdown of CAPN9 was able to potently suppress TGFβ1-induced expression of αSMA and faithfully recapitulated the effects of calpain inhibitors on EMT.

These data begin to suggest that induction of tissue-specific calpain isoforms, rather than increased function of the ubiquitously expressed ones, might be a fundamental mechanism underlying TGFβ-induced EMT.

A)



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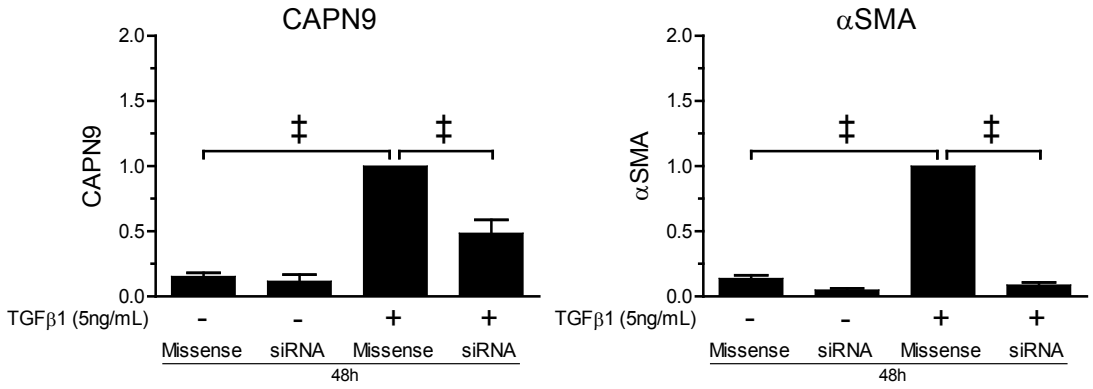
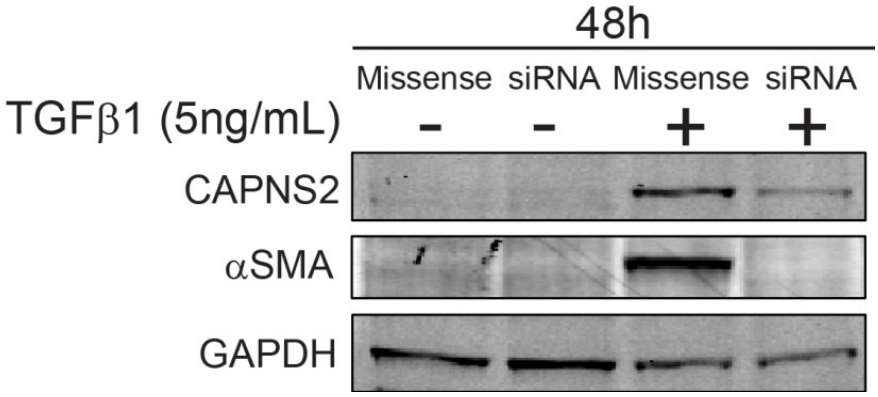


Figure 17: Western blot-assessment of a marker of TGFβ1-induced EMT after RNAi-mediated knockdown of *Capn9*. NMuMG (epithelial) cells were transfected with siRNA targeting *Capn9* or control siRNA 24 hours before treatment with TGFβ1 (5ng/mL). Cells were harvested 48 hours after treatment with TGFβ1. B) Protein levels were corrected for loading by GAPDH and normalized to the levels of present in samples transfected with control siRNA and treated with TGFβ1. Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

Knockdown of CAPNS2 inhibits TGFβ1-induced EMT

Like CAPN9, CAPNS2 was not expressed in NMuMG cells under steady state conditions (Figure 18). In contrast, treatment with TGFβ1 was able to induce expression of CAPNS2 and the mesenchymal marker αSMA. Knockdown of CAPNS2 in TGFβ1 treated cells was able to completely block expression of αSMA. We hypothesize that the stronger effect of CAPNS2, compared to CAPN9, may be due to variable efficiency of RNAi suppression and/or dosage dependence with regard to EMT. Alternatively, CAPNS2's may have the ability to form heterodimers with other calpain isoforms. Although CAPN1, CAPN2, and CAPN9 have been the only isoforms described to heterodimerize with CAPNS1 (Ravulapalli, Campbell et al. 2009), CAPNS2 may be able to form heterodimers with isoforms that are not CAPNS1 partners. These data may provide the first evidence for a unique function for CAPNS2, and warrant additional studies on its role within the calpain superfamily.

A)



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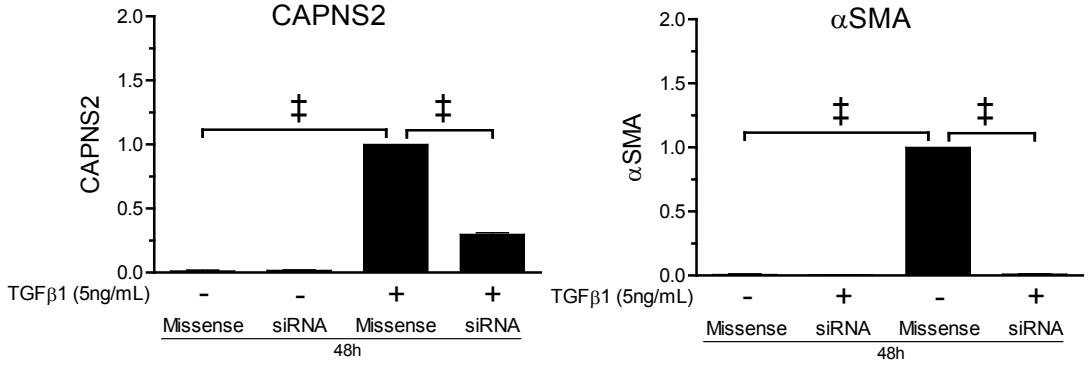


Figure 18: Western blot-assessment of a marker of TGFβ1-induced EMT after RNAi-mediated knockdown of *Capns2*. A) NMuMG (epithelial) cells were transfected with siRNA targeting *Capns2* or control siRNA 24 hours before treatment with TGFβ1 (5ng/mL). Cells were harvested 48 hours after treatment with TGFβ1. B) Protein levels were corrected for loading by GAPDH and normalized to the levels of present in samples transfected with a control siRNA and treated with TGFβ1. Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

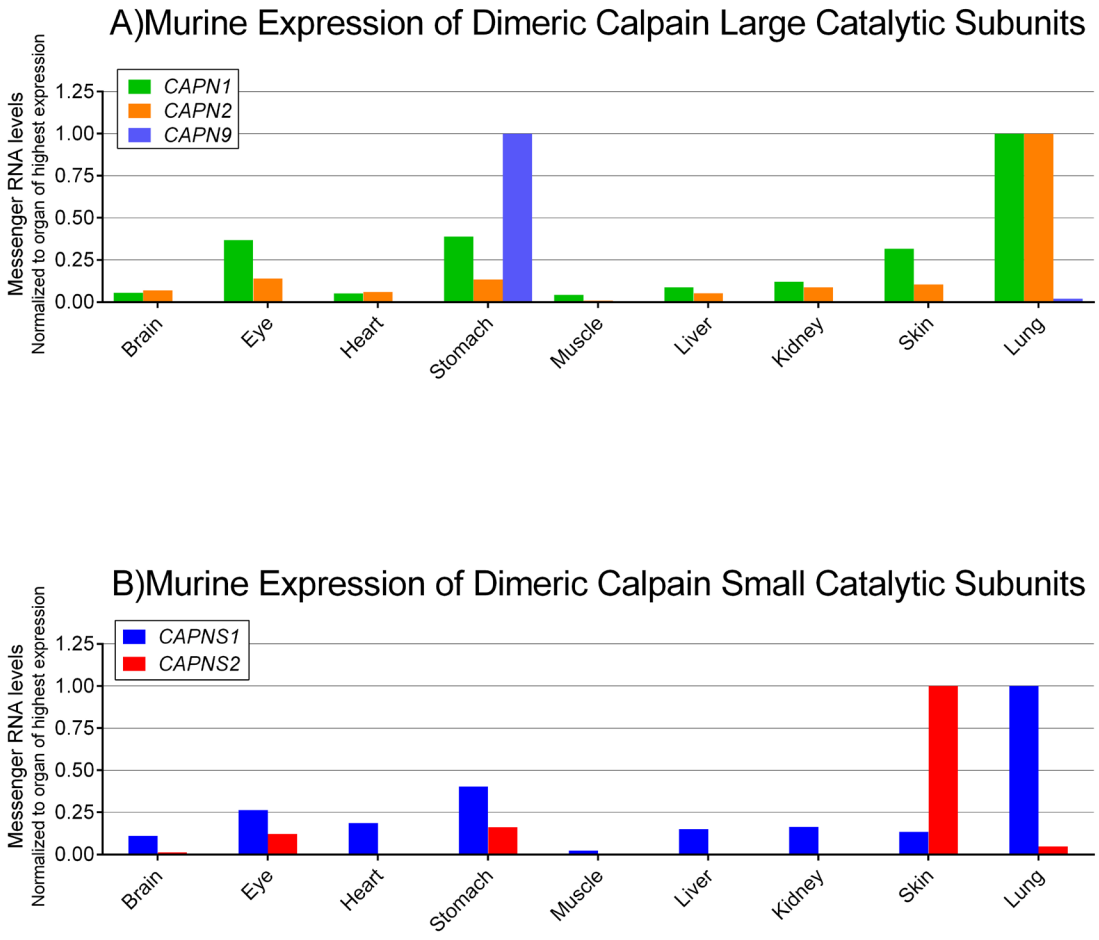


Figure 19: Expression of dimeric calpain isoforms in various mouse organs as assessed by quantitative PCR. A) Large catalytic calpain subunit B) Small regulatory subunit. Normalized to organ of highest expression.

Knockdown of CAPNS2 inhibits TGFβ1-induced EnMT

To further characterize the role of CAPNS2 during mesenchymal transition, we tested the effect of its inhibition on EnMT in Primary Porcine Aortic Valve Endothelial Cells (PAVEC). Quantitative PCR analysis revealed that treatment with TGFβ1 for 48 hours induced expression of mRNA encoding for several mesenchymal marker proteins consistent with induction of EnMT, such as *ACTA2* (encoding αSMA) and *VIM* (encoding vimentin), *MMP2* (encoding MMP-2*)*, and *MMP9* (encoding MMP9); and decreased expression of *CDH1* (encoding E-cadherin*;* Figure 20). Treatment with TGFβ1 induced expression of *CAPNS2*, and knockdown by siRNA targeting *CAPNS2* resulted in suppression of mesenchymal marker proteins and maintenance of *CDH1* expression. While overexpression of calpastatin in PAVECs during EnMT only partially suppressed expression of *MMP2/9* and *VIM* (Figure 13), knockdown of *CAPNS2* was able to completely block TGFβ1-induced *MMP2/9* expression and more strongly inhibit expression of *VIM* (Figure 20)

Immunofluorescence of PAVECs undergoing TGFβ1-induced EnMT illustrates that knockdown of *CAPNS2* by siRNA was able to block downregulation of E-cadherin and suppress expression of vimentin (Figure 21). Morphologically, TGFβ1-induced EnMT caused the PAVECs to disassociate and adopt a spindle shape appearance, which was suppressed by inhibition of *CAPNS2* expression (Figure 21). These data indicate that in endothelial cells, expression of the *CAPNS2* is induced by high TGFβ1 signaling and that the presence of CAPNS2 is critical EnMT. We hypothesize that CAPNS2 forms a

complex with CAPN9, and as a heterodimer, has activity that is required for TGFβ-induced EMT. The robust ability of calpain inhibitors to suppress TGFβ-induced mesenchymal transition in multiple cell types, limited expression of the critical calpain isoforms, and viability of the *CAPN9*$^{-/-}$ mice all strongly support targeting CAPN9 or CAPNS2 dependent activity for the treatment of TGFβ-mediated mesenchymal transition and associated pathologies, such as fibrosis. In later chapters, we will explore the effect of inhibiting CAPNS2 and/or CAPN9 on TGFβ1-induced mesenchymal transition in additional cell types and EMT-associated disease models.

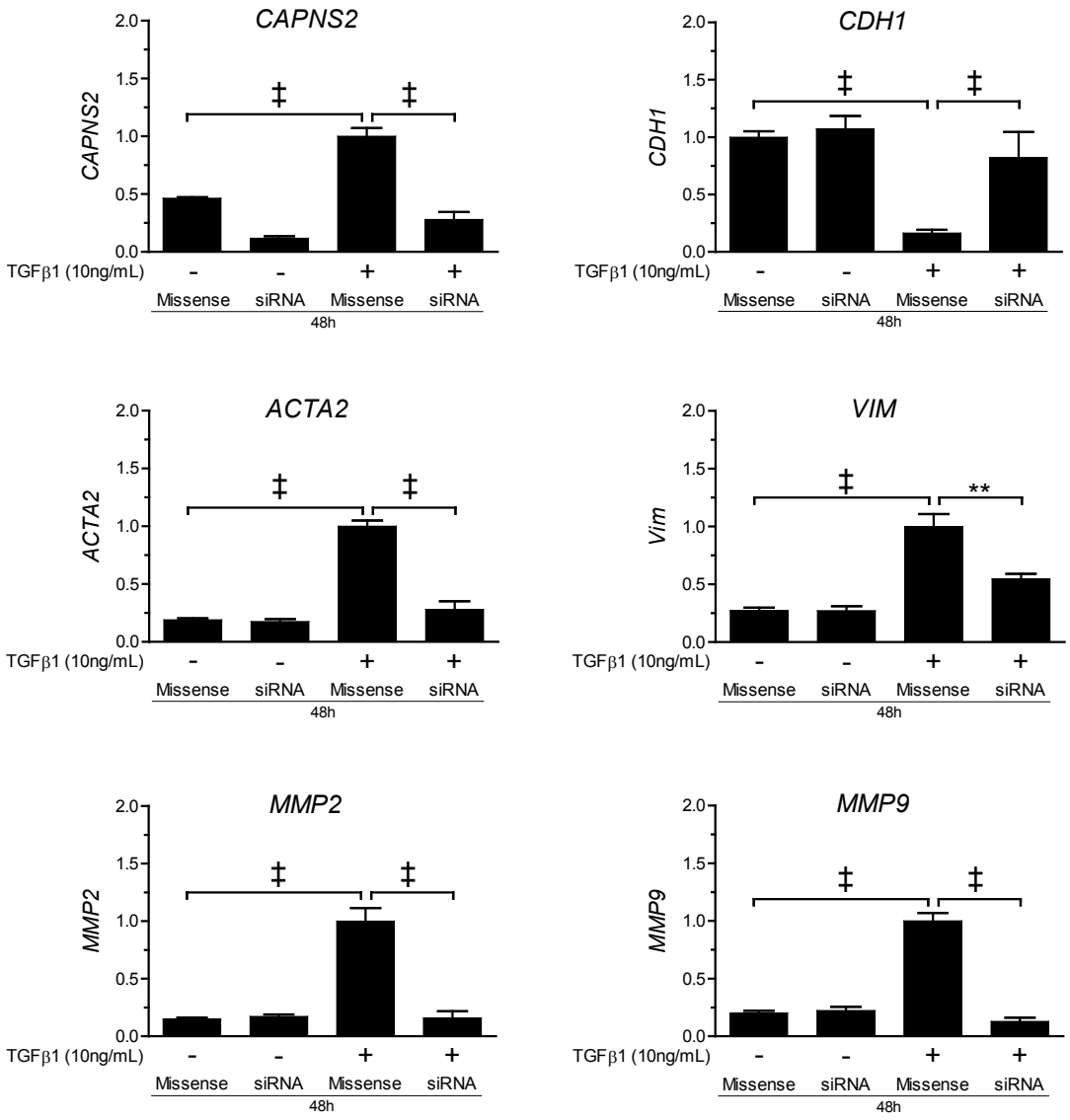


Figure 20: Quantitative PCR analysis of TGFβ1-induced expression of EnMT-genes in PAVEC (endothelial) cells after suppression of *CAPNS2* expression. Cells were transiently transfected with a siRNA targeting *CAPNS2* or control siRNA for 48 hours, and then treated with TGFβ1 (10ng/mL) for an additional 48 hours. Messenger RNA expression levels were corrected for loading to *GAPDH* and normalized to the average value of samples transfected with control siRNA and treated with TGFβ1. Results reflect experiments performed in biological quadruplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001.

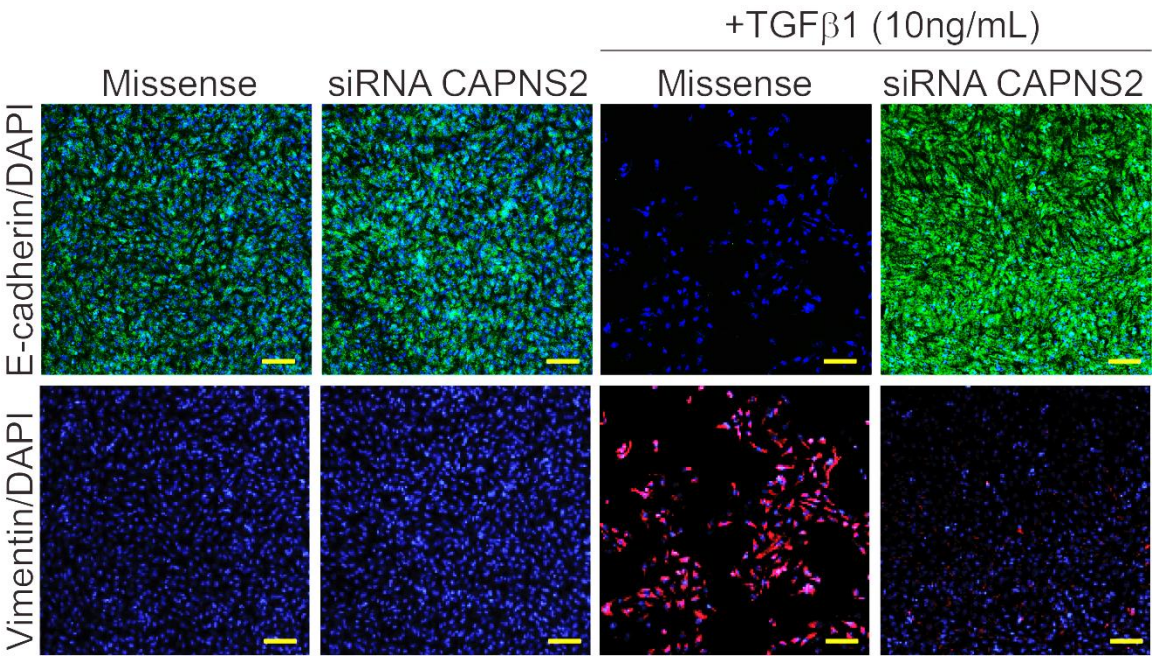


Figure 21: Knockdown of *Capns2* inhibits morphological changes during TGFβ1-induced EnMT. PAVEC (endothelial) cells were transfected with a siRNA targeting *CAPNS2* or a control siRNA for 48h, followed by treated with TGFβ1 (10ng/mL) for 48 hours. Cells were stained for E-cadherin (green), Vimentin (Red), and DAPI (Blue). Scale bar: 100μm.

Chapter 5

Calcineurin as a Critcal Calpain Substrate for EMT

Introduction:

The experiments presented in the previous chapters supports a model by which TGFβ signaling-dependent expression and activation of a CAPN9/CAPNS2 heterodimer is required for TGFβ-induced mesenchymal transition in a variety of in *vitro* models. Once activated, the CAPN9/CAPNS2 heterodimer can cleave and thus modify the activity of critical substrates, to activate a specific transcriptional program to mediate transition to a mesenchymal state. To identify possible critical mediators, we initially cross-referenced known calpain substrates with proteins involved in mesenchymal transition. During this literature search, one study provided evidence for a possible downstream mechanism consistent with our hypothesis.

A study by Molkentin and colleagues employed a genome-wide screen to identify mediators of fibroblast-to-myofibroblast transition (FMT) in cardiac fibroblasts (Davis, Burr et al. 2012). In this study, expression of either interleukin-6 (IL-6) or the calcium channel TRPC6, in cultured cardiac fibroblasts, was sufficient to induce expression of αSMA and FMT. Additionally, primary dermal fibroblasts derived from *Trpc6*$^{-/-}$ mice were resistant to TGFβ1-induced αSMA expression and FMT (Davis, Burr et al. 2012). In vivo, *Trpc6*$^{-/-}$ mice subjected to a dermal punch biopsy exhibited impaired wound healing/closure compared to wild-type littermates and significantly fewer αSMA positive myofibroblasts on the

boarder of the wound (Davis 2012), both of which are consistent with suppression of FMT (Yan 2010).

Moreover, these authors also showed that TGFβ1 treatment induced expression of *Trpc6* (but not other TRPC family members) in cultured cardiac fibroblasts and this was mediated through a p38 MAPK-dependent mechanism, independent of SMAD signaling (Davis, Burr et al. 2012). Chronic exposure to TGFβ1 (48 hours) or overexpression of TRPC6 in cardiac fibroblasts, resulted in increased cytosolic calcium concentration, but acute treatment with TGFβ1 did not, suggesting a gene expression-dependent mechanism (Davis, Burr et al. 2012).

Our data in NMuMG epithelial cells also found a significant increase in calpain activity 48 hours after TGFβ treatment, but not at 24 hours (Figure 1) and similarly suggests that transcriptional changes are required. However, some studies have specifically implicated the TRPM7 divalent cation channel, rather than TRPC6, in mesenchymal transition (Figure 7). Increased expression of TRPM7 is associated with in increased calpain activity (Su, Agapito et al. 2006), and cardiac fibrosis (Du, Xie et al. 2010). It is possible that TRPM7 and TRPC6 might facilitate calcium influx by working in a hetero-oligomeric complex, as has been shown to occur for other members of this class of channels (Alessandri-Haber, Dina et al. 2009, Ma, Qiu et al. 2010, Davis, Burr et al. 2012).

TRPC6-mediated Ca^{2+} influx has been shown to activate the calcineurin-Nuclear Factor of Activated T-cells (NFAT) pathway (Kuwahara, Wang et al. 2006). Calcineurin is a heterodimeric protein phosphatase, composed of a

catalytic A-chain (CnA) and a regulatory B-chain (CnB), both of which are required for activation of NFAT transcription factors.

Calcineurin was first identified as the target of the widely used immunosuppressive agent cyclosporine and as a critical mediator for activation of immune T cells (Clipstone and Crabtree 1992, Schreiber and Crabtree 1992). In addition to its role in T cell activation, calcineurin has also been shown to play a role in many other cellular processes including signaling during development of the immune, nervous, cardiovascular, and musculoskeletal systems (Aramburu, Heitman et al. 2004). Inhibitors of calcineurin, such as cyclosporine, have been used as potent immunosuppressive agents to treat autoimmune disorders and prevent organ transplant rejection, but are also associated with severe side effects.

In response to an increase in intracellular Ca^{2+} concentration, the regulatory CnB subunit of calcineurin binds Ca^{2+} and catalyzes a conformational change in the CnA subunit that exposes the calmodulin binding site (CaM) (Yang and Klee 2000). Binding of Ca^{2+}-bound calmodulin to the CaM site of CnA displaces the auto-inhibitory domain of CnA from the catalytic active site resulting in activation of calcineurin phosphatase activity (Rumi-Masante, Rusinga et al. 2012, Ye, Feng et al. 2013).

A major downstream target of calcineurin phosphatase activity is the NFAT family of transcription factors. Calcineurin-dependent dephosphorylation of NFAT uncovers the nuclear localization signal and allows its translocation to the nucleus (Clipstone and Crabtree 1992) where it can induce expression of a

variety of targets in concert with other transcription factors (Crabtree and Olson 2002). There is significant redundancy in function between the four NFAT isoforms based on the relatively benign phenotypes of single isoform knockout mice, but more severe phenotypes of mice lacking multiple combinations of isoforms (Crabtree and Olson 2002).

Expression of constitutively active calcineurin, lacking the C-terminal auto-inhibitory domain, (Hubbard and Klee 1989, O'Keefe, Tamura et al. 1992) in cultured cardiac fibroblasts was sufficient to induce expression of αSMA and induce FMT, while overexpression of the endogenous calcineurin inhibitor cain (Lai, Burnett et al. 1998) was able to block TGFβ1-dependent and TRPC6-overexpression-induced FMT (Davis, Burr et al. 2012). Inhibition of NFAT using the pharmacological inhibitor VIVIT was also able to block TGFβ1-dependent and TRPC6 overexpression-induced FMT (Davis, Burr et al. 2012).

Interestingly, calpains are known to cleave within the auto-inhibitory domain of calcineurin, resulting in its activation (Wang, Roufogalis et al. 1989, Shioda, Moriguchi et al. 2006). Additionally, calpains have been reported to cleave and inactivate the endogenous calcineurin inhibitor cain (a.k.a. cabin1) in response to ionomycin-induced calcium influx (Kim, Jo et al. 2002). Treatment with a calpain inhibitor, was able to suppress ionomycin-induced cleavage of cain and prevent activation of calcineurin (Kim, Jo et al. 2002). Based on these findings which support a role for calcineurin-mediated signaling in mesenchymal transition, and a role for calpain activity in activation of calcineurin phosphatase

activity, we hypothesized that calcineurin might be a critical CAPN9/CAPNS2 substrate during TGFβ-induced EMT.

Methods:

Western Blot Analysis

Western blot analysis was carried out using the same procedures outlined in Chapter 2 of this thesis. Membranes were probed with antibodies for rabbit anti-Calcineurin from StressMarq (SPC-175D) at a 1:1000 dilution, and goat anti-GAPDH from Santa Cruz Biotechnology (sc-20457) at a 1:5000 dilution.

Results:

Calcineurin as a Potential Calpain Substrate During Mesenchymal Transition

NMuMG cells under steady state conditions express both the full length and cleaved forms of calcineurin, as assessed by western blot (Figure 22). The signal for the cleaved form of calcineurin is significantly higher compared to the full-length form, suggesting that calcineurin is predominantly in its cleaved form under these conditions (or possibly, that the antibody has a greater affinity for the cleaved form). Treatment of these cells with TGFβ greatly increases the level of cleaved calcineurin, and this increase is abrogated by siRNA-mediated CAPN9 knockdown, suggesting that CAPN9 activity is required for processing of calcineurin in response to TGFβ. Additional experiments, targeting the other

dimeric calpain isoform subunits will be required to further characterize calcineurin processing by this mechanism.

In Figure 23, we propose a calcineurin-mediated mechanism to explain the function of calpains during TGFβ-induced mesenchymal transition. In this pathway, high TGFβ signaling causes increased levels of intracellular calcium through an indirect mechanism, possibly involving TRP channels, as suggested by previous studies (Davis, Burr et al. 2012). Calcium signaling by itself can activate calcineurin phosphatase activity through the classical, and reversible, calmodulin-dependent mechanism. Our data shows that acute exposure to TGFβ1 can induce expression of CAPN9/CAPNS2 (Figure 17,18), and that CAPN9 is necessary for TGFβ1-induced cleavage of calcineurin (Figure 22). This results in generation of an active phosphatase that would retain its activity, despite a subsequent decrease in intracellular calcium concentration. This cleaved form of calcineurin would not be subject to regulatory mechanisms that govern active, full-length calcineurin. As previously mentioned, calpains have been shown to cleave the endogenous calcineurin inhibitor cain (Kim, Jo et al. 2002), further potentiating its ability to activate the calcineurin pathway. Activation of calcineurin by either of these signaling mechanisms eventually leads to expression of NFAT-responsive genes, including cytokines such as IL-6 (Nilsson, Sun et al. 2007). It is worth noting that the same genetic screen used to identify TRPC6 as an inducer of FMT, also identified IL-6 (Davis, Burr et al. 2012). Interleukin-6 has also been recognized as one of the few signaling molecules that can robustly induce myofibroblast differentiation that is

independent from TGFβ signaling (Hinz 2007) and would be in line with our proposed mechanism by placing it downstream of TGFβ. *IL-6*$^{-/-}$ mice exhibit delayed wound healing/closure in response to a punch biopsy (Lin, Kondo et al. 2003, Gallucci, Lee et al. 2006), suggesting an impairment of non-developmental EMT. In the next chapter, we will explore the effect of inhibiting calpain isoforms that we have demonstrated to be critical for TGFβ-induced EMT in *in vivo* models of fibrosis.

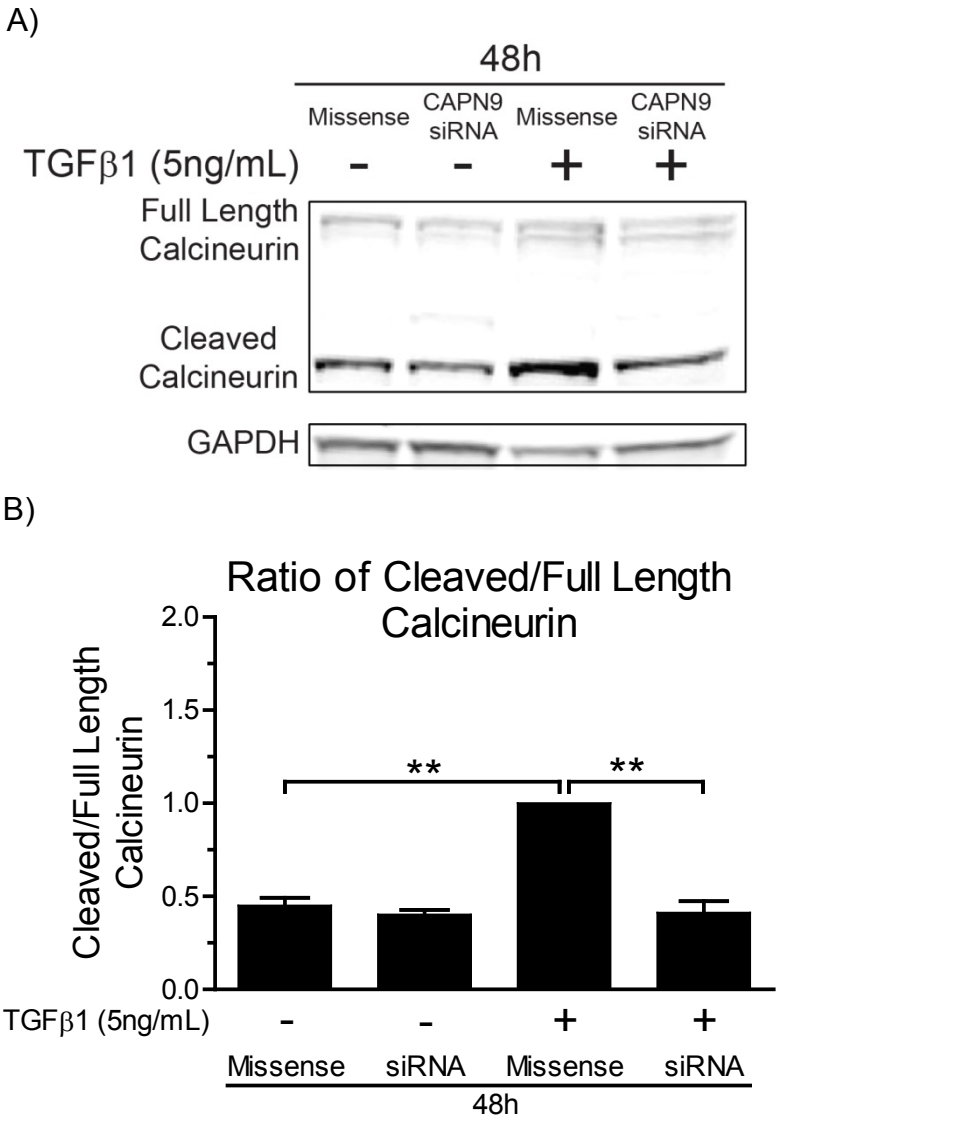


Figure 22: Western blot-assessment characterizing the effect of CAPN9 knockdown on calcineurin cleavage during TGFβ1-induced EMT. A) NMuMG (epithelial) cells were transfected with siRNA targeting *Capn9* or a missense control, 24 hours prior to treatment with TGFβ1 (5ng/mL) for 48 hours . B) Results are normalized to the levels present in samples transfected with control siRNA and treated with TGFβ1. Results reflect experiments performed in biological triplicate. Statistics were calculated using Student's t-test: *p<0.05 **p<0.01 †p<0.005 ‡p<0.001. C) Calcineurin domain structure in relation to calpain cleavage site.

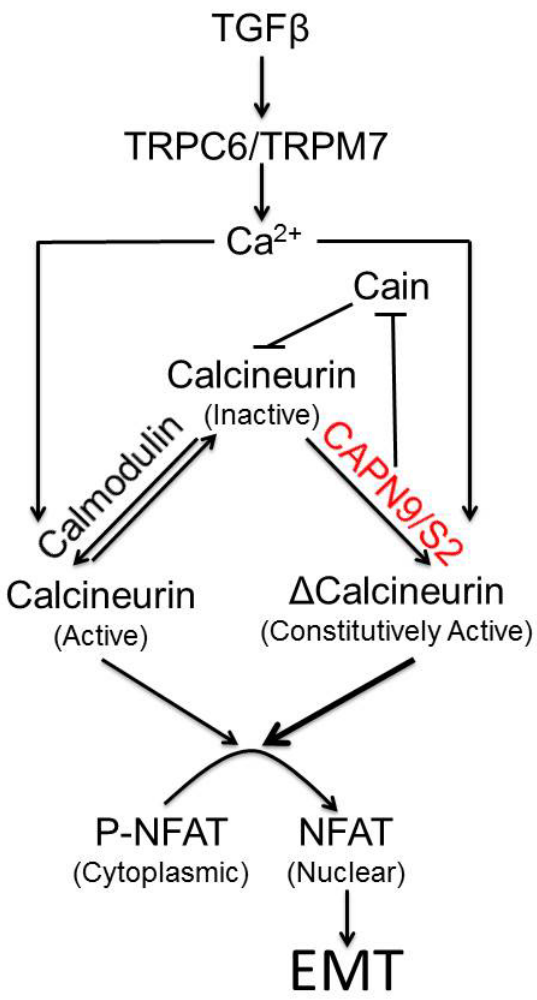


Figure 23: Proposed mechanism for calpain-dependent cleavage of calcineurin during TGFβ-induced mesenchymal transition. High TGFβ signaling induces expression of TRPC6/TRPM7 ion-channels, which results in an increase in cytosolic Ca^{2+} concentration. In the canonical pathway, calmodulin binds Ca^{2+} and activates calcineurin. We show that high TGFβ signaling also induce expression of the CAPN9/CAPNS2 heterodimer, which can cleave the endogenous calcineurin inhibitor cain (resulting in its inactivation), and also cleave calcineurin within its auto-inhibitory domain (resulting in its constitutive activation;ΔCalcineurin). Activated calcineurin de-phosphorylates NFAT, allowing it to enter the nucleus and alter gene transcription to promote EMT

Chapter 6

Inhibition of the CAPN9/CAPNS2 Heterodimer in *in Vivo* Models of Fibrosis

Introduction:

Although no ideal animal model for human fibrotic diseases exists, several attempts have been made to model the development of fibrosis in mouse in order to test the efficacy of anti-fibrotic therapies. The most commonly used models are bleomycin-induced lung fibrosis (Lee, Reese et al. 2014), carbon tetrachloride-induced liver fibrosis (Paquet and Kamphausen 1975), and unilateral ureteral obstruction-induced kidney fibrosis (Sharma, Mauer et al. 1993).

Bleomycin-induced lung fibrosis has been one of the most widely adopted models (Moore and Hogaboam 2008, Degryse and Lawson 2011). Bleomycin is an antibiotic that is clinically used as an intravenous chemotheraputic agent. Pulmonary fibrosis and (to a lesser degree) skin fibrosis are the most common side effects associated with bleomycin administration in humans. Relatively low levels of bleomycin hydrolase, the enzyme that metabolizes bleomycin, in these tissues might explain their susceptibility to fibrosis in response to this drug (Lazo and Humphreys 1983, Takeda, Masuda et al. 1996). Likewise, mouse strains that express higher levels of bleomycin hydrolase in the lungs, such as the Balb/C strain, have been found to be resistant to bleomycin-induced lung fibrosis (Filderman and Lazo 1991).

It is thought that bleomycin-mediated degradation of DNA and consequent cell death (Mirabelli, Ting et al. 1982, Tounekti, Kenani et al. 2001) leads to secretion of pro-inflammatory cytokines (Tumor Necrosis Factor α, IL-1β, IL-13,

gp160, and IL-6) (Cavarra, Carraro et al. 2004) and the generation of pro-fibrotic myofibroblasts (Alipio, Jones et al. 2011, Aoyagi-Ikeda, Maeno et al. 2011, Zhao, Qin et al. 2014, Chen, Ye et al. 2015). In this model, myofibroblasts have been shown to be derived from mesenchymal transition of multiple cell types, including epithelial cells (Tanjore, Xu et al. 2009), endothelial cells, (Hashimoto, Phan et al. 2010)), and fibroblasts (Chen, Zhang et al. 2013).

In order to assess the effect of inhibiting *Capn9* or *Capns2* expression in fibrosis development, we used various strategies to test the effect of isoform-specific suppression of calpain activity in the development of bleomycin-induced lung fibrosis.

Currently there are no reported pharmacological calpain inhibitors that are specific for a single isoform. Therefore, in order to achieve isoform specific inhibition of calpain activity, we used intratracheal instillation of siRNA to specifically target *Capns2 mRNA* expression (Bonifazi, D'Angelo et al. 2010, Zafra, Mazzeo et al. 2014) and a *Capn9*$^{-/-}$ mouse model (Hata, Abe et al. 2010) to specifically assess a requirement for CAPN9.

Methods

Administration of siRNA targeting *Capns2* and intratracheal bleomycin in mice.

Eight-week-old C57Bl6 male mice were purchased from Jackson Laboratories (000664). Mice were maintained on an *ad libitum* diet and standard

12 hour light/dark cycle in accordance with protocols set forth by Johns Hopkins University Institutional Animal Care and Use Committee (Protocol MO12M124). A SMARTpool of Accell siRNAs targeting *Capns2* (sequence M-014858-00-0010) or Accell siRNA non-targeting missense control (sequence D-001206-13-50) were purchased from GE Healthcare. Small interfering RNAs were resuspended in 1x GE Healthcare siRNA buffer (B-002000-UB-100) at a concentration of 2.5 nanomoles/50 µL. Bleomycin sulfate was purchased from Sigma (B5507-15UN) and dissolved in PBS for a final concentration of 0.05U/50µL.

Mice were anesthetized with 250 µL of Avertin solution (2,2,2 tribromoethanol dissolved in tert-amyl alcohol),diluted to 2.5% in PBS. For drug delivery, mice were first placed on a 45° inclined plane and intubated with a PennCentury microsprayer aerosolizing syringe (MSA-250) (Bivas-Benita, Zwier et al. 2005) with the assistance of a laryngoscope. Animals were first treated with siRNA targeting *Capns2* or a non-targeting missense control at a dose of 2.5 nanomoles in 50 µL of siRNA buffer solution. The following day, lung fibrosis was initiated in mice by treatment with intratracheally delivered 0.05U bleomycin in 50uL saline vehicle, for a final dose of 2U/kg as previously described (Lee, Reese et al. 2014). Direct intratracheal delivery of bleomycin was chosen to parallel the intratracheal delivery of siRNA and to maximize delivery to the lungs. Mice were analyzed 14 days after initial bleomycin injection and after treatment with siRNA targeting *Capns2* or a non-targeting missense control according to the schedule shown in Figure 24. The dosing regimen was designed based on current data describing siRNA delivery into the lungs of mice (Yueksekdag,

Drechsel et al. 2010) and our own experience with *in vitro* experiments. Mice were euthanized with an intraperitoneal injection of 200 µL 10% ketamine solution, and perfused with PBS. For western blot analysis, lungs were isolated and sonicated in RIPA buffer. Samples were spun at 20,000g for 15 minutes and supernatant isolated for western blot analysis as described in Chapter 2. Membranes were probed using goat anti-Collagen I from Southern Biotech (1310-01), goat anti-Collagen III from Southern Biotech (1330-01), and goat anti-GAPDH from Santa Cruz Biotechnology (sc-20457). For quantitative PCR analysis, RNA from lungs was isolated and processed as described in Chapter 4.

Systemically Delivered Bleomycin-Induced Fibrosis Model in *Capn9*$^{-/-}$ mice

Capn9$^{-/-}$ mice have been previously described (Hata, Abe et al. 2010). Cryopreserved sperm from *Capn9*$^{+/-}$ mice (RBRC04790) was obtained from the Experimental Animal Division of RIKEN and rederived in mice on a C57Bl6 background by the Johns Hopkins University School of Medicine Transgenic Core Laboratory. DNA from mice was extracted using the Qiagen DNeasy blood and tissue kit (69506). Mice were genotyped by PCR using the Bio-Rad iProof^{TM} High Fidelity PCR kit (172-5331) with GC buffer. PCR primers were synthesized by Integrated DNA Technologies and using sequences and thermal cycling conditions as previously described (Hata, Abe et al. 2010).

Eight-week-old C57Bl6 male mice from Jackson labs, or *Capn9*$^{-/-}$ mice were treated with systemic bleomycin by osmotic pump as previously described (Lee, Reese et al. 2014). In brief, animals were anesthetized with 4% isofluorane

by gas mask under a ventilated hood. Mice were shaved and a small lateral incision was made between the scapulae. Each mouse was implanted with an Alzet Micro-osmotic pump (Model 1007D) filled with vehicle saline or a bleomycin solution prepared to achieve a dose of 100U/kg over a period of 7 days (Lee, Reese et al. 2014). The wound was closed using tissue clips and mice were allowed to recover. Pumps were explanted 10 days later to prevent release of toxic salts from the pump (Lee, Reese et al. 2014) and the wound was resealed with tissue clips. Lung fibrosis, which under this method of delivery, is primary localized of the subplural regions (Lee, Reese et al. 2014), was allowed to develop for an additional 25 days following removal of the pump. At the end of the trial, mice were euthanized with an intraperitoneal injection of 200 µL 10% ketamine and perfused with PBS. Right lung lobes were tied off with a suture and the trachea was cannulated with a blunted syringe that inflated the left lobe with warm 0.7% agarose in PBS. The left lung was allowed to solidify and fixed in 10% formalin overnight. Fixed lungs were then transferred to 70% ethanol solution and sent to AML Laboratories (Baltimore, MD) for paraffin embedding, 10 µm sectioning, and Masson Trichrome staining. Lungs were imaged on a Nikon Elipse 80i scope using a 10x objective lens under bright field and captured using Nikon NIS Elements software.

Results:

RNAi-mediated knockdown of CAPNS2 suppresses intratracheal bleomycin-induced pulmonary fibrosis in a murine model.

In order to assess the anti-fibrotic potential of CAPNS2 inhibition, we monitored the levels of lung fibrosis in mice in which pulmonary fibrosis was induced by intratracheal administration of bleomycin concomitantly with control siRNA or siRNA targeting *Capns2* (Figure 25). Western blot analysis of lungs from bleomycin-treated mice that had received control siRNA showed high levels of collagen I and collagen III compared to saline controls, although variable levels of Collagen I expression were observed. This variability may reflect uneven distribution of bleomycin upon delivery, causing some lung lobes to have more fibrosis relative to others within the same mouse.

Lungs from bleomycin-treated mice that had received siRNA targeting *Capns2* showed significantly lower levels of collagen I and collagen III compared to lungs derived from mice treated with control siRNA. Indeed, they expressed levels of collagen I and collagen III similar to those found in the lungs of saline-treated animals. CAPNS2 was not present in lungs from vehicle treated mice but was highly expressed in lungs from mice treated with control siRNA and bleomycin. In contrast, lungs from mice treated with siRNA targeting *Capns2* and bleomycin showed significantly lower levels of CAPNS2 compared to mice treated with control siRNA and bleomycin, confirming successful knockdown of our target.

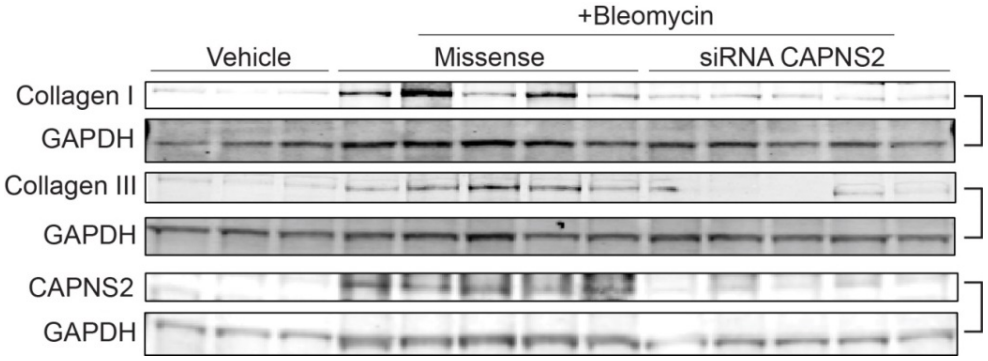
Assessment of *Capn9* expression by qPCR shows that this calpain isoform is greatly induced in lungs from bleomycin-treated mice when compared to saline-treated controls (Figure 25C); this is consistent with our previous observations (Figure 17) that, in most tissues, Capn9 is not expressed under physiological conditions, but it is induced by fibrotic stimuli such as TGFβ.

We hypothesized that the therapeutic effect of CAPNS2 knockdown could be attributed to a requirement for CAPNS2 for the formation of a functional proteolytic heterodimer between this subunit and CAPN9, whose activity is necessary for TGFβ-induced EMT (Figure 17). We thus set out to directly test the effect of knocking out *Capn9* on bleomycin-induced pulmonary fibrosis.

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
siRNA CAPNS2 or Missense Control	Bleomycin				siRNA CAPNS2 or Missense Control	
Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
		siRNA CAPNS2 or Missense Control				siRNA CAPNS2 or Missense Control
Day 15	Day 16					
	Harvest Lungs					

Figure 24: Dosing schedule for RNAi-mediated knockdown of *Capns2* during intratracheal bleomycin-induced lung fibrosis. Mice were pretreated with siRNA targeting *Capns2* or a non-targeting missense control before delivery of bleomycin. In addition, small interfering RNAs (siRNA) were also delivered on days 6, 10, and 14. Lungs were harvested on day 16.

A)



B)

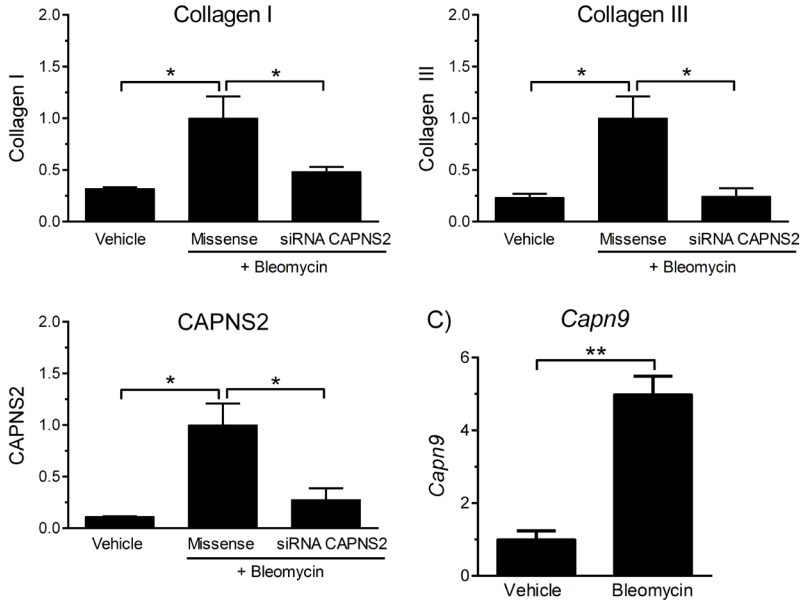


Figure 25: Knockdown of *Capns2* suppresses bleomycin-induced fibrosis in mice. Eight-week-old C57Bl6 mice were pretreated with intratracheal siRNA targeting *Capns2* or a control siRNA. The next day, mice received an intratracheal injection of bleomycin (2U/kg) in PBS solution. From this time, mice siRNA targeting *Capns2* or control siRNA every 3 days for a total of 3 additional doses as described in Figure 24. A) Protein isolated from lungs was analyzed by western blot for levels of Collagen I, Collagen III with GAPDH as a loading control. B) Western blots were quantitated and statistics calculated using Kruskal-Wallis non-parametric one-way analysis of variance. C) Quantitative PCR analysis of *Capn9* mRNA levels in lungs from bleomycin treated lungs compared to vehicle treated lungs. Messenger RNA levels were normalized to lungs derived from mice treated with vehicle.

Capn9$^{-/-}$ mice are protected from systemically delivered bleomycin-induced lung fibrosis .

Systemic delivery of bleomycin (via subcutaneous osmotic pump) allows for more uniform drug delivery compared to direct intratracheal instillation and results in subplural fibrosis, which is similar to scleroderma-associated lung fibrosis (Lee, Reese et al. 2014). We used this method to induce lung fibrosis in control mice and in mice in which CAPN9 had been genetically ablated (Hata, Abe et al. 2010).

Histological analysis by Masson Trichrome staining of lungs derived from control mice treated with systemic bleomycin reveals severe subplural fibrosis all along the perimeter of the lung (Figure 26). When these lungs were inflated with 0.7% agarose solution, the edges of the lungs failed to expand, while the center of the lung had relatively higher compliance and quickly enlarged.
In stark contrast, lungs from *Capn9*$^{-/-}$ mice treated with bleomycin showed little to no fibrosis by histology and were virtually indistinguishable from wild type mice treated with saline. There were also significantly fewer infiltrating cells in the lungs from bleomycin-treated *Capn9*$^{-/-}$ mice or saline-treated wild type mice, as compared to bleomycin-treated wild-type animals. These data support the notion that CAPN9 is a critical mediator of bleomycin-induced fibrosis, and that CAPN9 might represent an exciting new target for anti-fibrotic drugs, especially considering that we saw no evidence of a compensatory mechanism in response to *Capn9* deficiency, under both physiological and chronic pathological conditions.

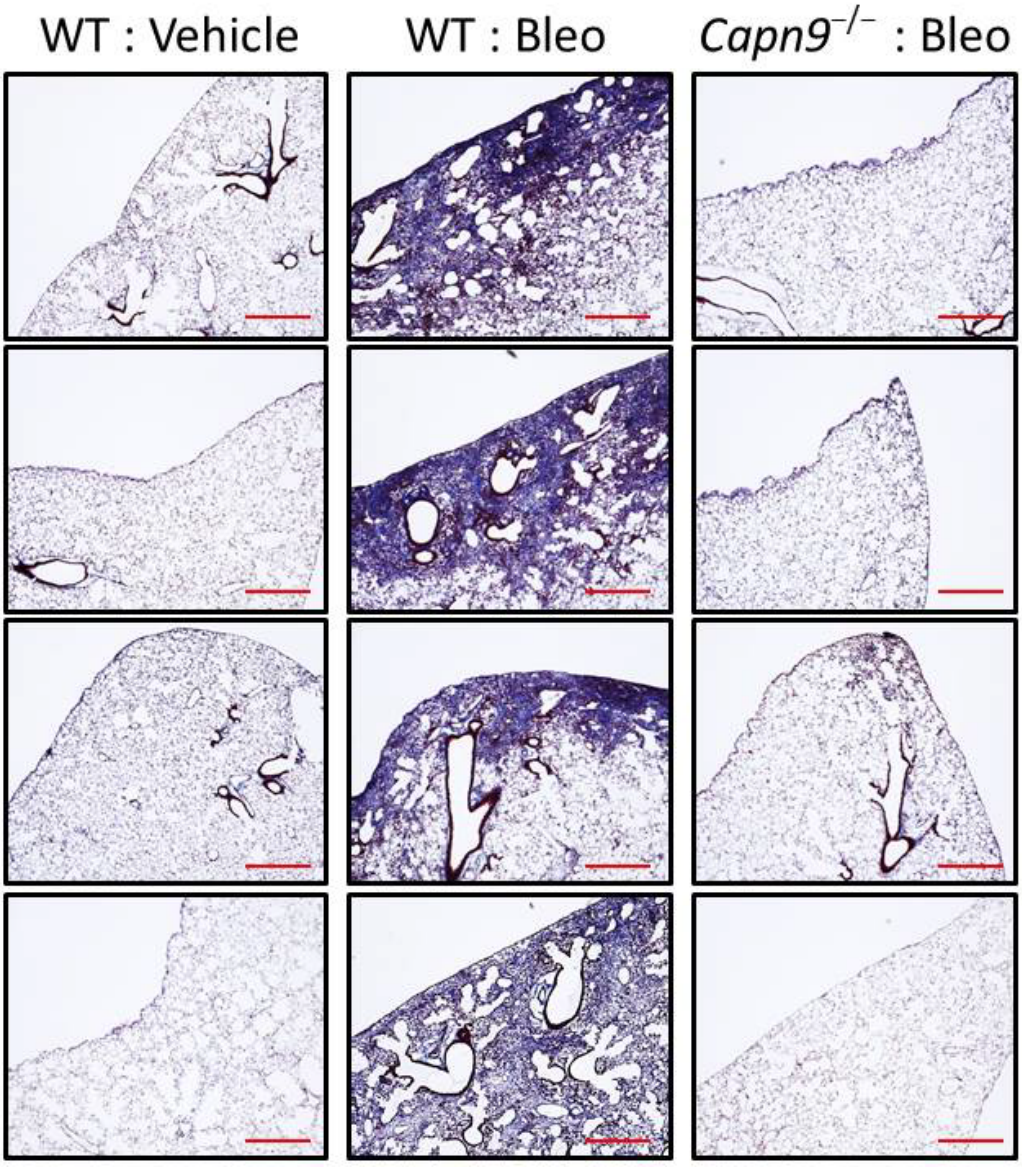


Figure 26: *Capn9*$^{-/-}$ mice are resistant to systemically delivered bleomycin-induced lung fibrosis. Eight-week-old C57Bl6 wild-type or *Capn9*$^{-/-}$ mice were implanted with subcutaneous osmotic pumps that delivered 100U/kg bleomycin in a saline solution over a period of 7 days, was explanted 3 days later, and allowed to develop fibrosis for an additional 25 days. Control C57Bl6 wild-type mice were implanted with pumps delivering saline. Lungs were formalin fixed, paraffin embedded, sectioned and stained for fibrosis using Masson Trichrome. Scale Bar: 500μm

Chapter 7

Discussion

Epithelial-to-Mesenchymal Transition (EMT) is a complex biological process that has evolved to fulfill critical functions that are essential for both embryonic development and for tissue repair in response to injury (i.e. wound healing). Development-associated EMT purposefully functions to regulate the generation of diverse cell types in order to facilitate tissue/organ formation and does not involve fibrosis, inflammation, or obligate adoption of an invasive phenotype (Kalluri and Weinberg 2009). EMT during gastrulation has been shown to be primarily regulated by WNT signaling (Liu, Wakamiya et al. 1999, Skromne and Stern 2001), which is mediated through the TGFβ superfamily proteins NODAL and Vg1 (Collignon, Varlet et al. 1996, Shah, Skromne et al. 1997, Varlet, Collignon et al. 1997, Skromne and Stern 2002, Chea, Wright et al. 2005).

In contrast, the EMT process that is initiated in response to tissue injury is closely linked to inflammation and leads to the generation of invasive myofibroblasts; in the presence of constant inflammatory stimuli the initial healing response can cause excessive fibrosis and lead to tissue and organ dysfunction (Kalluri and Weinberg 2009). Upon injury, immune cells, most prominently macrophages (Kalluri and Weinberg 2009), infiltrate the site of injury and release pro-inflammatory cytokines such as IL-1β, Tumor Necrosis Factor α, and IL-6 along with the pro-fibrotic cytokine TGFβ (Mirza and Koh 2015). These macrophages also secrete chemo-attractant cytokines and matrix degrading

enzymes (MMP2/9) that facilitate further immune cell infiltration, release of cytokines such as TGFβ, and inflammation(Strutz, Zeisberg et al. 2002, Kalluri and Weinberg 2009). High TGFβ signaling has been shown to be critical in the development of most forms of fibrosis (Branton and Kopp 1999).

Past attempts to use TGFβ antagonists to treat EMT-associated diseases have found that this class of drugs had severe side effects, probably caused by the fact that, in addition to its role in fibrosis, TGFβ signaling also serves other important functions such as growth inhibition (Laiho, DeCaprio et al. 1990), suppression of inflammation (Sheng, Chen et al. 2015), and hematopoiesis (Fortunel, Hatzfeld et al. 2000).

Our data has repeatedly shown that inhibition of calpain activity does not affect early TGFβ signaling events (Figure 2,3,11), but can potently inhibit TGFβ-induced expression of fibrosis-associated genes (Figure 5). We were initially surprised to learn that TGFβ-induced EMT utilizes calpain isoforms that are not normally expressed in the relevant progenitor cell types, but rather are rapidly and potently induced by TGFβ. In retrospect this appears to be a remarkably elegant and efficient strategy for nature to tightly regulate a process that is occasionally necessary and productive (as in the case of wound healing), but can in often prove deleterious if left unchecked. Restriction of this EMT-promoting activity to calpains that are uniquely expressed in response to an overt fibrogenic stimulus such as TGFβ prevents activation of the EMT pathway in response to other physiological stimuli that result in increased intracellular Ca^{2+} concentration and normally result in activation of other constitutively expressed calpains. In this

light, it may also make sense that *Capn9* and *Capns2* are normally only highly expressed in the gastrointestinal tract and skin (Figure 19), respectively, two tissues that are subject to excessive wear-and-tear and show a remarkable capacity for rapid repair and regeneration.

Consistent with the notion that CAPN9 and CAPNS2 serve specialized functions in the context of EMT and fibrosis, the *Capn9*$^{-/-}$ mouse is able to fully develop and is physiologically normal (Hata, Abe et al. 2010), yet is incredibly resistant to bleomycin-induced lung fibrosis compared to wild-type mice (Figure 26). Based on these data, we believe that calpain inhibitors designed to specifically suppress activity of CAPN9/CAPNS2 would be a potentially ideal class of drugs for targeting fibrosis-associated EMT exclusively. Side effects would be limited because such a drug would not affect the physiological functions of TGFβ or the enzymatic activity of constitutively expressed calpains.

Myofibroblasts have been firmly implicated in the majority of fibrotic conditions (Estes, Vande Berg et al. 1994). Various cell types can undergo mesenchymal transition to give rise to pro-fibrotic myofibroblasts (Tanjore, Xu et al. 2009, Hashimoto, Phan et al. 2010, Chen, Zhang et al. 2013). Importantly, we have shown that calpain inhibitors are able to inhibit myofibroblast formation from several major sources, including epithelial cells (Figure 2,3,11), fibroblasts (Figure 8), and endothelial cells (Figure 13, Figure 20). Our data also show that calpain activity is necessary to maintain a myofibroblast state, suggesting that a targeted calpain inhibitor could be curative for patients in which fibrosis is already established.

Our data suggest that during TGFβ-induced EMT, the pro-fibrotic activity of CAPN9/CAPNS2 is at least in part mediated by activation of the phosphatase calcineurin. Although this might suggest that calcineurin inhibitors (e.g. cyclosporin, tacrolimus) might be used as anti-fibrotic drugs, the severe side effects associated with these drugs, such as hypertension and renal toxicity, make their use for the treatment of fibrosis less desireable (Morton and Powell 2000). In a small clinical study, 13 scleroderma patients were given cyclosporine to treat skin fibrosis and half noticed a small improvement in skin thickness, but side effects became so severe that it led to 12 of the patients withdrawing from the trial (Morton and Powell 2000). These drugs have been shown to inhibit a constitutively active form of calcineurin (O'Keefe, Tamura et al. 1992) that is very similar to that generated upon cleavage by calpains. Their modest but dose-limited effect on skin fibrosis (Morton and Powell 2000) suggests that a calpain inhibitor could have greater efficacy to treat fibrotic conditions due to its predicted lower toxicity.

Based on the restricted expression of CAPN9/CAPNS2 in the gastrointestinal tract and skin (Figure 19), we believe that a drug targeting CAPN9/CAPNS2 would not suppress the immune system. In addition, *Capn9*$^{-/-}$ mice do not exhibit an immuno-compromised phenotype (Hata, Abe et al. 2010).

Currently, there are no effective therapies to treat the numerous fibrotic disorders that affect a significant number of the world's population. Our work has identified a new pathway that is critically involved in a cellular mechanism linked to numerous disease processes and possesses most of the characteristics

required in an ideal drug target. It is our hope that this work will lead to the development of safer therapeutic drugs for the treatment of fibrosis and other EMT-associated pathologies.

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Education and Training

2007-2015 Ph.D. Johns Hopkins University School of Medicine, Cellular and Molecular Medicine Program
Doctoral Dissertation: Targeting CAPN9/CAPNS2 Activity as a Therapeutic Strategy for the Treatment of Transforming Growth Factor Beta-Induced Mesenchymal Transition and Associated Pathologies
Mentor: Hal Dietz, M.D

2006-2007 M.S. University of California San Diego, Department of Anesthesiology
Masters Thesis: Studying the Role of Cytosolic Phosphplipase A2 in Nociception
Mentor: Tony Yaksh, Ph.D.

2002-2006 B.S. University of California San Diego, Division of Biological Sciences
Major: Biochemistry and Cell Biology

Teaching Experience

2006 Teaching Assistant, University of California San Diego, Cell Biology Lab Course
2003-2004 Lab Assistant, University of California San Diego, Lab of Roger Y. Tsien,Ph.D.

Peer-Reviewed Publications

1. Holm TM, Habashi JP, Doyle JJ, Bedja D, Chen Y, van Erp C, Lindsay ME, **Kim D**, Schoenhoff F, Cohn RD, Loeys BL, Thomas CJ, Patnaik S, Marugan JJ, Judge DP, Dietz HC (2011) Noncanonical TGFβ signaling contributes to aortic aneurysm progression in Marfan syndrome mice 2011. Science 332(6027):358-61 doi: 10.1126. PMID 214936862.

2. **Kim DH**, Fitzsimmons B, Hefferan MP, Svensson CI, Wancewicz E, Monia BP, Hung G, Butler M, Marsala M, Hua XY, Yaksh TL. (2008) Inhibition of spinal cytosolic phospholipase A(2) expression by an antisense oligonucleotide attenuates tissue injury-induced hyperalgesia. Neuroscience 153(3):1077-87 doi 10.1016. PMID 18511207.

Invited Presentations

1. **Kim D**, Gould R, Butcher J, Dietz H. (2014) Targeting Calpains: A Therapeutic Strategy for the treatment of TGFβ Mediated Mesenchymal Transition and Associated Pathologies. 64th Annual Meeting of the American Society of Human Genetics, San Diego, CA, October 21, 2014.

2. **Kim D**, Patel N, Lindsay M, Goldmuntz E, John A, Garbarini J, Dietz H. (2010) Mechanistic Insights Regarding Filaminopathies and Aneurysm. 8th International Symposium on Marfan Syndrome, Warrenton, VA, September 13, 2010.

3. **Kim D**, Patel N, Lindsay M, Goldmuntz E, John A, Garbarini J, Dietz H. (2009) Calpain as a Therapeutic Target in Inherited Aortic Aneurysm: Lessons from Rare Mendelian Disorders. 59th Annual Meeting of the American Society of Human Genetics, Honolulu, HI, October 24, 2009.

Abstracts

1. Patel ND, **Kim DH**, Lindsay ME, Holm TM, John A, Garbarini J, Goldmuntz E, Dietz HC. FIlamin A Mutations Cause Tetralogy of Fallot (ToF) with Ascending Aortic Aneurysm. 58th Annual Meeting of the American Society of Human Genetics, Philadelphia, PA, November 13, 2008.

2. **Kim DH**, Svensson CI, Fitzsimmons BL, Wancewicz EV, Monia BP, Huang G, Butler M, Yaksh TL, Hua XY. Expression and Regulation of Cytosolic Phospholipase A2 in Nociception. 36th Annual Meeting of the Society for Neuroscience, Atlanta, GA, October 15, 2006.

Provisional Patents

Kim DH, Dietz HD. Targeting the CAPN9/CAPNS2 Heterodimer as a Therapeutic Strategy for the Treatment of TGFβ-Mediated Mesenchymal Transition and Associated Pathologies. United States Patent Application 62/046,383 filed on September 5, 2014.

Society Memberships

2009, 2014	American Society for Human Genetics
2008	Society for Neuroscience

Awards

2014	Semifinalist, Charles J. Epstein Award, American Society for Human Genetics
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